Universidade do Minho
Escola de Engenharia

Pedro Tiago Santos Silva

Production of bacterial reserve compounds from industrial wastewaters

Outubro de 2013

Production of bacterial reserve compounds from industrial wastewaters
Pedro Tiago Santos Silva
Uminho | 2013

Universidade do Minho
Escola de Engenharia

Pedro Tiago Santos Silva

Production of bacterial reserve compounds from industrial wastewaters

Dissertação de Mestrado
Mestrado em Bioengenharia

Trabalho efetuado sob a orientação da
Doutora Maria Alcina Alpoim de Sousa Pereira
e da
Professora Doutora Maria Madalena dos Santos Alves

Outubro de 2013

DECLARAÇÃO

Nome: Pedro Tiago Santos Silva

Endereço eletrónico: pedro.ssilva6@gmail.com

Número do Bilhete de Identidade: 13751675

Título da tese: Production of bacterial reserve compounds from industrial wastewater

Orientadores: Doutora Maria Alcina Alpoim de Sousa Pereira e Professora Doutora Maria Madalena dos Santos Alves

Ano de conclusão: 2013

Designação do Mestrado: Mestrado em Bioengenharia

Universidade do Minho, ___/___/_____

Assinatura: _____

ACKNOWLEDGMENTS

The current work was developed in two different places. The first part took place from September 2012 to February 2013 at Avecom NV, Ghent, Belgium. The second one started in February 2013 and ended in October 2013 and took place at the Center of Biological Engineering of Minho University, Braga, Portugal, where this dissertation was written. During both periods of time, numerous people contributed, in one way or another, to the development of the work.

In first place, I thank Professor Madalena Alves for giving me the opportunity to do an Erasmus Placement, more specifically doing an internship at Avecom NV and University of Minho, the host institution giving the financing support through an Erasmus grant.

Along all my stay in Ghent, the work was strongly supported by the supervision and orientation by Professor Emeritus Willy Verstraete, to whom I am personally grateful. He always showed interest in my work, accompanied it closely and carefully, suggesting improvements and enhancing the whole experiment practice with his knowledge and scope of the subject. My acknowledgment to all my colleagues at Avecom, particularly Lutgart Stragier, Nuno Sardo, Rik Daneels and also Tijs Vanslambrouck who helped me in adjusting to the new company and the new country. My appreciation for the way they made me feel welcome in Belgium.

To my supervisor, Professor Alcina Pereira, I thank her for the opportunity to continue developing my work. I would like to emphasize all the support, knowledge, motivation and availability that she gave me during this work.

To Rita Castro, I thank her for all the support, friendship, patience and knowledge, without her help it would not have been possible to finish this work. To José Carlos, I thank him for all the knowledge and help that he gave me with the statistical software used in the current work. My acknowledgments to all my colleagues from the BRIDGE Group at the Center of Biological Engineering of Minho University who helped me with the work and gave me their friendship.

At a personal level, I thank my friends for the good moments and advices. In addition, to Stephanie Biesemans, who accompanied me along this journey closely, I thank her for the great experiences, feelings, and all the support she gave me categorically.

Last but not least, I thank my family, who was always present. Thanks for all the understanding, lessons and support you gave to me and still show me unconditionally. You made the completion of my course easier and to you this dissertation is dedicated.

A todos, um grande e sincero obrigado!

Aan iedereen, een grote en oprechte dankuwel!

ABSTRACT

This thesis aimed to study the accumulation of reserve compounds such as polyhydroxyalkanoates (PHAs), triacylglycerols (TAGs) and wax esters (WEs), using mixed bacterial cultures. These reserve compounds can be produced from wastewaters derived from the agro-industrial sector. Valorization of these residues together with production of potentially high-added value products can turn out to be a process economically viable and simultaneously beneficial for the environment. The reserve compounds mentioned can be used in oleochemical and biofuel industry, being an alternative to the fossil fuels. Nowadays, the dependence of fossil fuels for energy production is evidently not sustainable.

In the first part of the work, 25 kg of biomass enriched in PHA was produced at pilot scale. The PHA content of the final product was about 20%. Afterwards, this novel product was subjected to tests in order to analyze their potential application. Therefore, the challenge proposed at the beginning of the work was successfully performed using a full set-up at pilot scale for PHA production, considering all the general steps needed for implementation at industrial scale. During the fermentation step, the volatile suspended solids (VSS) concentration was between 2.98 and 4.76 g/L with yields in a range of 0.21 and 0.30 g PHA/g COD. At the end of the fermentation, the mixed culture used could accumulate between 37 to 38% of PHA using acetic acid as carbon source.

In the second part of the work, other reserve compounds were produced besides PHAs due to low volatile fatty acids content in the substrate used. However, no analysis was available to confirm it at Avecom NV. From batch experiments performed and an overall balance labored, promising results were obtained. With the (open) mixed culture and the complex culture media used, it was found that an adaptation phase to select the organisms capable of accumulating reserve compounds is not necessary. The culture media used consisted of vegetable processing waste streams with shortage of nutrients i.e., the amount of nitrogen and phosphate was very low compared to the amount of carbon oxygen demanded (COD). So, if a good VSS yield was obtained, a good storage compound yield would be obtained as well because there were no nutrients to allow growth of the biomass. Best VSS yields, 0.40-0.43 g VSS/g $CODt_{removed}$, were obtained when the inoculum was subjected to only 8 hours of fill-up.

The third part included optimization of the biosynthesis and accumulation of reserve compounds from a wastewater containing mineral oil. Several conditions were tested, according to an experimental design, using a response surface methodology (RSM). Three independent variables were tested, which were COD concentration, nitrogen concentration and cultivation time. Overall, the culture used was able to synthesized and accumulate three different reserve compounds, namely PHAs, WEs and TAGs. The highest PHA accumulation, according to the response surface model applied to the obtained data, was achieved for 14 g COD/L, 0.06 g N/L with a cultivation time of 57 hours. In these conditions the PHA content in the biomass would be of about 8.6%.

RESUMO

Esta tese teve como objectivo estudar a acumulação de compostos de reserva tais como: polihidroxialcanoatos (PHAs), triacilgliceróis (TAGs) e ésteres de cera (ECs), utilizando culturas bacterianas mistas. Estes compostos de reserva podem ser produzidos a partir de águas residuais do sector agro-industrial. A valorização destes resíduos, juntamente com a produção de produtos de alto valor acrescentado pode vir a ser um processo economicamente viável e, simultaneamente, benéfico para o meio ambiente. Os compostos de reserva mencionados podem ser utilizados na indústria oleoquímica e dos biocombustíveis, sendo uma alternativa aos combustíveis fósseis. Hoje em dia, a dependência de combustíveis fósseis para produção de energia não é, claramente, sustentável.

Na primeira parte do trabalho, foram produzidos 25 kg de biomassa enriquecida em PHA à escala piloto. O teor de PHA do produto final foi cerca de 20%. Posteriormente, este novo produto foi submetido a testes, tendo em vista uma potencial aplicação. Assim sendo, o desafio proposto no início do trabalho foi realizado com sucesso recorrendo á instalação completa de um sistema em escala piloto para produção de PHA, considerando todas as etapas gerais necessárias para a implementação à escala industrial. Durante a etapa de fermentação, a concentração de sólidos suspensos voláteis (SSV) foi entre 2,98 e 4,76 g/L com um rendimento na gama de 0,21 e 0,30 g PHA/g COD. No final da fermentação, a cultura mista usada acumulou entre 37 e 38% de PHA, utilizando ácido acético como fonte de carbono.

Na segunda parte do trabalho, foram produzidos outros compostos de reserva para além dos PHAs devido ao baixo conteúdo de ácidos gordos voláteis do substrato utilizado. No entanto, nenhuma análise estava disponível na Avecom NV para o confirmar. A partir de experiências realizadas em batch e um balanço geral realizado, foram obtidos resultados promissores. Com a cultura mista e os meios complexos de cultura usados, verificou-se não ser necessário uma fase de adaptação para seleccionar os organismos capazes de acumular compostos de reserva. Como meios de cultura foram usadas águas residuais provenientes do processamento de vegetais com falta de nutrientes, isto é, em que a quantidade de azoto e fosfato é muita baixa comparada com a quantidade de carência química de oxigénio (CQO). Assim, se um bom rendimento de VSS fosse obtido, também seria obtido bom rendimento de compostos de reserva acumulados porque não havia nutrientes suficientes para se obter um crescimento da biomassa. Os melhores rendimentos de VSS foram obtidos quando o inóculo foi submetido apenas a 8 horas de fase de enchimento: 0,40-0,43 g VSS/g $CQOt_{removido}$.

A terceira parte incluiu a optimização da biossíntese e acumulação de compostos de reserva a partir de uma água residual que contém óleos minerais. Várias condições foram testadas, conforme um desenho experimental, utilizando a metodologia de superfície de resposta (RSM). Três variáveis independentes foram testadas, nomeadamente a concentração de CQO, a concentração de azoto e o tempo de incubação. Em geral, a cultura utilizada foi capaz de sintetizar e acumular três diferentes compostos de reserva, nomeadamente os PHAs, ECs e TAGs. De acordo com o modelo de superfície de resposta ajustado aos dados obtidos, a maior acumulação de PHA verifica-se para 14 g CQO/L, 0,06 g/L de azoto, com um tempo de cultura de 57 horas. Nestas condições, o teor de PHA na biomassa seria cerca de 8,6%.

TABLE OF CONTENTS

Bv: Organic loading rate

Bx: Sludge loading rate

C/N: carbon/nitrogen

CDW: Cell dry weight

CoA: Coenzyme A

COD: Chemical oxygen demand

CODs: Soluble chemical oxygen demand

CODt: Total chemical oxygen demand

d: Day

DGGE: Denaturing gradient gel electrophoresis

DM: Dry matter

DO: Dissolved oxygen

EBPR: Enhanced biological phosphorus removal

EDTA: Ethylenediaminetetraacetic acid

FA: Fatty acid

FAMEs: Fatty acid methyl-esters

GC: gas chromatography

GC-FID: Gas chromatography with flame ionization detector

NADP: Nicotinamide Adenine Dinucleotide Phosphate

MMC: Mixed microbial culture

P(3HB): Poly (3-hydroxybutyric acid)

P3HB4HB: Poly[(R)-3-hydroxybutyrate-*co*-4-hydroxybutyrate]

PBS: Poly-butylene succinate

PHA: Polyhydroxyalkanoates

PHA-DEG: Polydydroxyalkanoate-diethylene glycol

PHA_{LCL}: PHA long chain length

PHA_{MCL}: PHA medium chain length

PHA_{SCL}: PHA short chain length

PHB: Polyhydroxybutyrate

PHBHHx: Poly[(R)-3-hydroxybutyrate-*co*-(R)-3-hexanoate]

PHBV: Poly[(R)-3-hydroxybutyrate-*co*-(R)-3-hydroxyvalerate]

PHV: poly-3-hydroxyvalyrate

PLA: Polylactic acid

RSM: Response surface methodology

SBR: Sequencing batch reactor

SCFA: Short chain fatty acids

SDS: Sodium dodecyl sulfate

t: Time

TAG: Triacylglycerol

TAN: Total ammonium nitrogen

TKN: Total Kjeldahl nitrogen

TLC: Thin layer chromatography

TOC: Total organic carbon

TS: Total solids

TSS: Total suspended solids

UV: Ultraviolet

VFA: Volatile fatty acids

VFA-COD: content of Chemical Oxygen Demand in the Volatile Fatty Acids

VM: Volatile matter

VS: Volatile solids

VSS: Volatile suspended solids

WE: Wax ester

WWTP: Wastewater treatment plant

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1. RESEARCH GOALS

This thesis aims to study the production of reserve compounds such as polyhydroxyalkanoates, triacylglycerols and wax esters using mixed bacterial cultures. The following objectives were defined:

(I) Production and optimization at pilot scale of a novel product, biomass enriched in polyhydroxyalkanoates, for application trials. This study aimed at:

- The determination and further optimization of the most suitable production fermentation strategy at pilot scale
- The determination and further optimization of the most suitable analytical technique for quantification of the novel product
- The determination of the most efficient techniques of thickening and drying sludge
- The determination of the most suitable way to store the final product

(II) Optimization and production at lab scale of reserve compounds by the fermentation of organic side streams, to accomplish the following:

- The selection of the most suitable substrate from several organic side streams via batch tests
- Assess the need for a phase with selection of desirable organisms from an activated sludge inoculum

(III) Optimization of the biosynthesis and accumulation of reserve compounds from a wastewater containing spent lubricants and motor oils, to achieve the following:

- Determination of the most appropriate conditions (carbon and nitrogen concentrations and cultivation time) to accumulate polyhydroxyalkanoates
- Evaluation of the presence of neutral lipids, namely wax esters, fatty acids and triacylglycerols in biomass submitted to different conditions (carbon and nitrogen concentrations and cultivation time)

1.2. STRUCTURE AND ORGANIZATION OF THE DISSERTATION

In Figure 1.1 shows a schematic representation of the organization of this thesis.

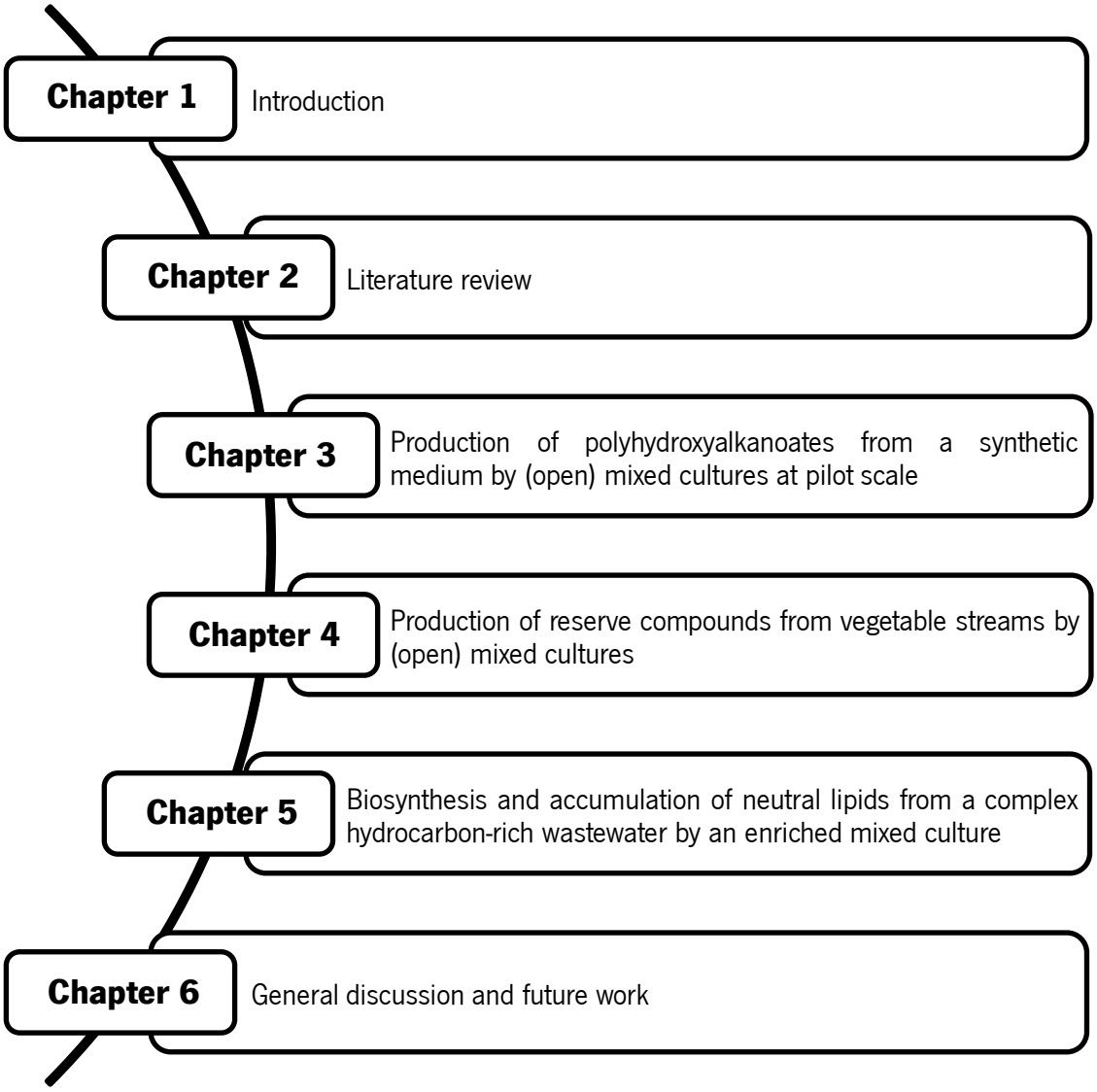

Figure 1.1: Schematic organization of this thesis.

CHAPTER 2

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. BIOTECHNOLOGICAL RELEVANCE OF BACTERIAL OILS

For millennia, human societies depended on biological resources for energy and materials. Plant material was combusted for heat, used for clothing and building materials, while animal power was harnessed for transportation [1]. Despite that, society in the early 21st century appears to be dependent of its fundamental source of materials and energy. Petroleum, the fuel that has been driving modern society for one century, is showing signs of scarcity [2]. Our current dependence on fossil fuels for energy generation is clearly not sustainable. Fossil fuels such as crude oil, natural gas or coal are non-renewable. The demand is continuously increasing and will continue to do so as economic growth goes on in countries as big as China or India. However, the supplies are limited and the discovery of new reserves does not match the increasing needs. Furthermore, burning fossil fuels leads to a net increase in the atmospheric carbon dioxide, which is very likely that contributes to the global warming process, generating a growing concern [3]. This context leads us to choose renewable resources as biodiesel and bioethanol. These fuels make up about 90% of the biofuel market [4, 5].

The continuously increasing demand and utilization of the so called "first generation biodiesel" that occurred in the last 15 years has increased the price of several foodstuffs, and this situation has led to the need of discovery of non-conventional sources of oils, that could be subsequently converted into biodiesel [6-9]. Specifically, according to the ''US Standard Specification for biodiesel'' (ASTM 6751-02), biodiesel is defined as ''a fuel that is composed of mono-alkyl esters of long-chain fatty acids deriving from vegetable oils or animal fats'' [10]. This definition is also acceptable in the European Union specification concerning biodiesel (EN 14214). Stricter regulations define biodiesel as fatty acid methyl-esters – FAMEs [10, 11].

Biodiesel is considered compatible with the existing fuel infrastructure, non-toxic, and has superior combustion characteristics than fossil diesel. Moreover, in 2008, the global production was 12.2 million tons [5]. Currently, this industrial biodiesel production is based almost exclusively on the chemical transesterification of triacylglicerols (TAGs) from vegetable oils employing methanol [5]. Nowadays, TAGs for biodiesel production are almost exclusively produced by agriculture. The main crops for TAGs production are rapeseed in Europe, oil palm trees in South East Asia, and soya in North America. However, TAG production using

microorganisms has also been developed. Oleaginous bacteria are promising candidates for biotechnological production of TAGs from renewable resources [12]. Production by bacteria gives a greater flexibility in comparison to agriculture production for different reasons such as: short life cycle, less labor required, independence of the season, climate and local, easier to scale up [13], access to genetic engineering to improve the yields of TAG production and a wide range of the substrates available [12]. Besides that, it can be found in the literature that several oleaginous microorganisms have the potential to remarkably growth and produce storage compounds from wastes and by-products of the agro-industrial sector [14-16]. These storage compounds can be TAGs, wax esters (WEs) or polyhydroxyalkanoates (PHAs). Valorization of those residues together with the production of potentially high-added value lipids could become a process economically viable and simultaneously beneficial for the environment [17].

Besides TAGs, WEs can also be used in biofuels being chemically converted [18]. WEs have several applications in cosmetic and food industry [19]. Moreover, WEs and TAGs inclusions as well as PHAs granules could be modified and used as a basis for the production of biodegradable, self-assembling nanoparticles, which could be useful for a wide range of sophisticated technical applications and in medicine [20].

Another interesting aspect of those bacterial oils is the possibility to be used by oleochemical industry. Currently, this kind of industry uses petrochemicals, i.e., oleochemicals obtained from fossil resources. From bacterial oils, the oleochemical industry could manufacture, for example, detergents, surfactants, adhesives, soaps, varnishes, paints, lubricants. Therefore, the oleochemical industry can generate those valuable products from renewable resources resulting in “natural” oleochemicals that is really important for their image to the public [21].

In particular, PHAs are polymers with interesting properties being considered an alternative to conventional petroleum derived plastics. They are biosynthetic, biodegradable and potentially biocompatible polymers [22-24]. PHAs were already used as biodegradable plastic materials for packaging, such as bags, containers, paper coatings and disposable items like razors, utensils, diapers and feminine hygiene products [25-27]. Currently, new applications are being studied to apply these biopolymers by Research & Development departments of companies as Avecom NV, Ghent, Belgium.

2.2. STORAGE COMPOUND ACCUMULATION IN BACTERIA

Many prokaryotes are able to accumulate large amounts of lipophilic compounds as inclusion bodies in the cytoplasm. Members of most genera synthesize polymeric lipids such as poly(3-hydroxybutyrate) (PHB) or other PHAs, whereas accumulation of TAGs and WEs in intracellular lipid-bodies is a property of only a few prokaryotes [28]. Accumulation of at least one type of storage lipid can be found in nearly all prokaryotes. Therefore, it is very likely that lipid accumulation is advantageous for survival in natural habitats and that the capability for their synthesis provides a strong advantage during evolution. The few exceptions of prokaryotes lacking the capability to accumulate lipids (for example, lactobacilli, *Enterobacteriaceae*, and methanogenic archaea) exist in nutrient-rich habitats, in which accumulation of lipids does not provide an advantage [28]. Afterwards, all these lipids act as storage compounds for energy and carbon needed for maintenance of metabolism and synthesis of cellular metabolites during starvation and in particular if growth resumes [28].

In most organisms, accumulation of neutral lipids is usually stimulated if a carbon source is present in the medium in excess and if the nitrogen source is limiting growth [29, 30]. Cellular growth is impaired under these conditions, and the cells utilize the carbon source mainly for the biosynthesis of neutral lipids [21]. Under conditions promoting growth, cells use the carbon source for the biosynthesis of fatty acids required for biosynthesis of phospholipids or other cellular components [31].

TAGs, WEs and PHAs represent ideal storage compounds, since they exhibit a low biological toxicity compared with free fatty or hydroxy fatty acids and are anhydrous and osmotically inert. Therefore, they do not affect the water balance of the cells [20]. Furthermore, accumulation of those storage compounds is more advantageous than proteins or carbohydrates, owing to their much higher caloric value, showing a minor grade of oxidation and their relative compactness [24, 32, 33]. In literature, the production of extracellular lipids has already been reported in some organisms. However, physiological role of lipid export is still unclear [31, 34, 35].

The metabolism leading to the storage compounds synthesis is quite unknown when it is correlated to the biosynthesis of PHAs, TAGs or WEs. Manilla-Perez *et al* [31] proposed a metabolic pathway to produce those storage compounds from hydrocarbons (Figure 2.1). Common precursors are shared in production of PHAs, TAGs and WEs as the acyl-CoA metabolites are obtained from a primary oxidation of the substrate. The crucial step to define the product obtained is in β-oxidation of free fatty acids cycle, as can be seen in Figure 2.1.

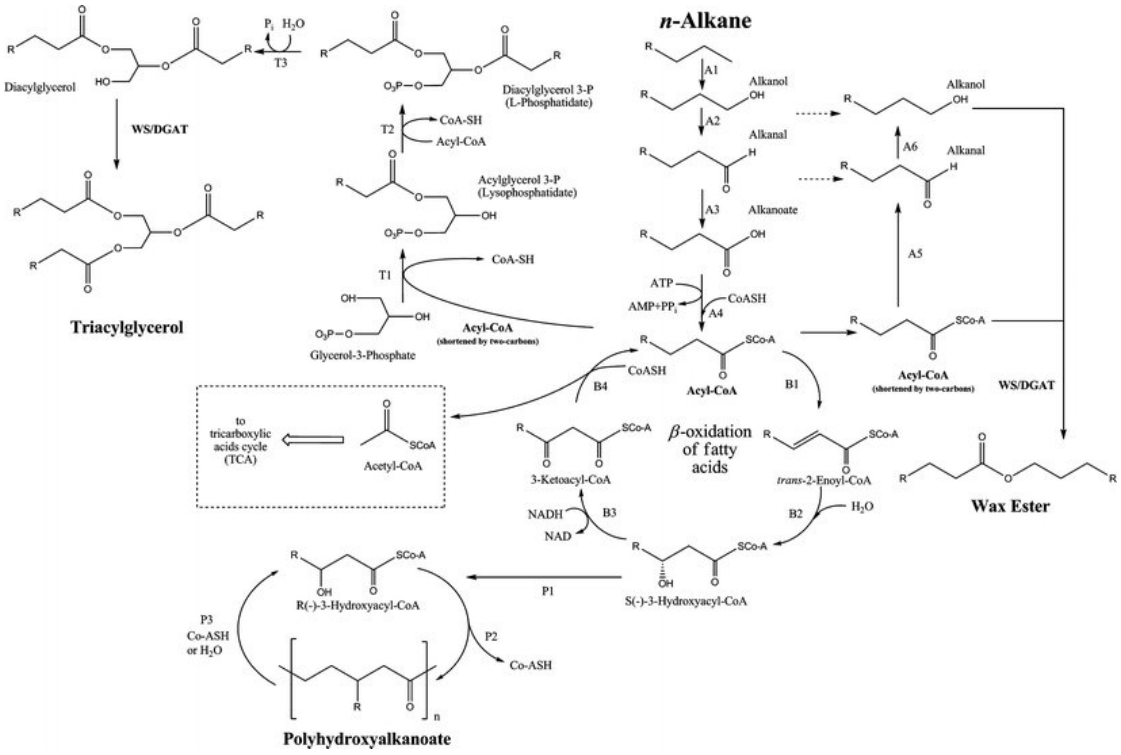

Figure 2.1: Proposed metabolic pathway, by Manilla-Perez *et al.* [31], to produce (lipophilic) storage compounds from hydrocarbons. Enzymes involved in the metabolic route are: alkane hydroxylase (A1), alcohol dehydrogenase (A2), alkanal dehydrogenase (A3), acyl-CoA synthase (A4), acyl-CoA dehydrogenase (A5), aldehyde reductase (A6) (alcohol dehydrogenase), acyl-CoA dehydrogenase (B1), enoyl-CoA hydratase (B2), 3-hydroxyacyl-CoA dehydrogenase (B3), 3-ketoacyl-CoA thiolase (B4), glycerol-3-phosphate acyltransferase (T1), 1-acylglycerol-3-phosphate acyltransferase (T2), phospatidate phosphatase (T3), WS/DGAT wax ester synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT), epimerase (P1), PHA synthase (P2), PHA depolymerase (P3).

2.3. TRIACYLGLYCEROLS

TAGs are formed by glycerol esters with long chain fatty acids (Figure 2.2). The oxidation of TAG produces the maximum yields of energy in comparison with other storage compounds such as carbohydrates or PHA, since the carbon atoms of the acyl moieties of TAG are in their most reductive form [21].

β-Oxidation of fatty acids contained in TAG produces large amounts of reducing equivalents, and Nicotinamide Adenine Dinucleotide Phosphate (NADP) must be regenerated. Therefore, β-oxidation can occur only under respiratory conditions. This is probably the reason why all TAG accumulating bacteria described so far are aerobic bacteria [21].

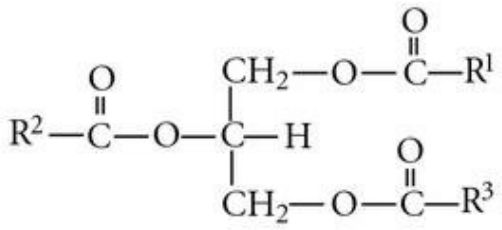

Figure 2.2: Chemical structure of TAG wherein R1, R2, R3 are an alkyl or an alkenyl, hydrocarbon chain of a fatty acid esterified on the glycerol backbone.

TAGs are currently produced at large scale by agriculture for synthesis of fatty acid methylesters that are exclusively used for biodiesel production [12].

In addition, Olukoshi and Packter [29] suggested TAG may also act as carbon source for the biosynthesis of antibiotics from acetyl-CoA or malonyl-CoA precursors. On the other hand, Solaiman *et al*. [36, 37] experimented TAGs as substrate for production of PHAs using bacteria as *Pseudomonas putida* and *Pseudomonas oleovorans*.

TAGs are not stored exclusively intracellularly. In literature, it is possible to find some microorganisms that manage to export small amounts of these lipids produced, such as *Alacanivorax jadensis T9 and Alcanivorax borkumensis SK2* [19].

2.4. WAX ESTERS

WEs are monoesters of long-chain fatty acids and alcohols (Figure 2.3). They form the basis of many commercial products, such as lubricants, printing inks, coating stuffs, cosmetics, and lacquers [35, 38]. *Acinetobacter* spp. are the most known organisms to accumulate wax ester as a storage material [39].

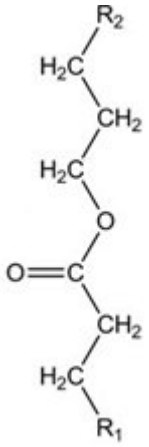

Figure 2.3: General structure of a wax ester.

At industrial scale, the production of WEs results from crude oil, mostly hexadecyl hexadecanoate [35], or a biological source as jojoba oil from the desert shrub, *Simmondsia californica* [40, 41]. Jojoba waxes are only available in limited quantities, because their production requires semiarid to arid land and appropriate climate [35].

The microbial production of wax ester has advantages over other biological sources since the wax ester composition can be controlled by the choice of starting material or growth conditions [42].

In bacteria, the enzyme responsible for catalyzing the esterification of a fatty acyl-coenzyme A (CoA) and a fatty alcohol is referred to as the wax ester synthase/acyl-coenzyme A:diacylglycerol acyltransferase (WS/DGAT) [43]. Together with the fatty acyl-CoA reductase and fatty aldehyde reductase enzymes which provide the requisite fatty alcohol, the WS/DGAT enzyme is proposed to produce wax esters in bacteria from the fatty acyl-CoA pool [31, 44]. One interesting feature of most WS/DGAT enzymes characterized to date is the fact that they are redundant and not specific, so they have a broad substrate range found for various substrates [45].

Generally, WEs are produced and stored intracellularly. However, *Alacanivorax jadensis*, *Alcanivorax SK2* and some species from *Acinetobacter* are able to export those reserve compounds to the medium [31, 35, 39, 42].

2.5. POLYHYDROXYALKANOATES

PHAs belong to a family of biopolyesters formed by fatty acids. Unlike polylactic acid (PLA) or poly-butylene succinate (PBS) that requires chemical polymerization, PHAs are the only type of bioplastics known to be completely synthesized by microorganisms as a form of storage material [46]. Upon synthesis, PHA is accumulated in the form of granules in the bacterial cell cytoplasm, as illustrated in Figure 2.4.

As bacterial storage compounds, PHAs are synthesized in times of imbalanced nutrient availability when a carbon source is available in excess but other nutrients have been depleted, as nitrogen, phosphate [47, 48] and/or oxygen limitations [49]. They form water-insoluble spherical inclusions or discrete granules inside the cell and can be mobilized when conditions are suitable. The number per cell and size can vary among different species [47]. Depending on the substrate and the synthesizing organism, PHAs are composed of monomers of different chain lengths – short-chain-length PHAs (PHA_{SCL}) and medium-chain-length PHAs (PHA_{MCL}) [46, 47, 50], as is illustrated in Figure 2.5. The former consist of monomers with 3-5 carbons atoms and

the latter contain monomers with 6-14 carbon atoms [51]. PHA_{SCL} is mainly found as poly (3-hydroxybutyric acid) P(3HB) [47].

(a) Alcaligenus latus (b) Pseudomonas oleovorans

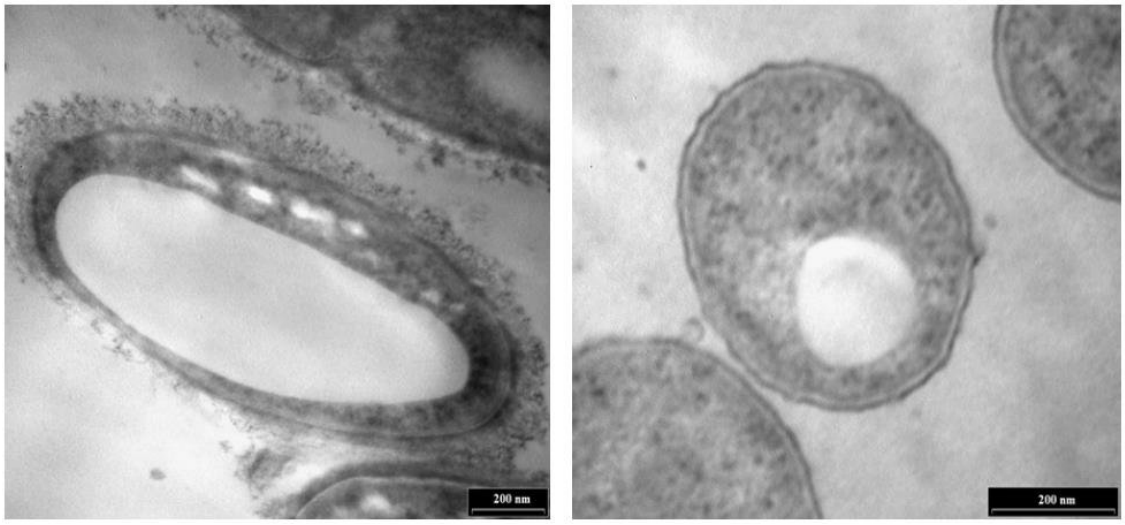

Figure 2.4: Transmission electron micrographs showing PHA inclusion bodies in (a) Alcaligenus latus (b) Pseudomonas oleovorans cells [52].

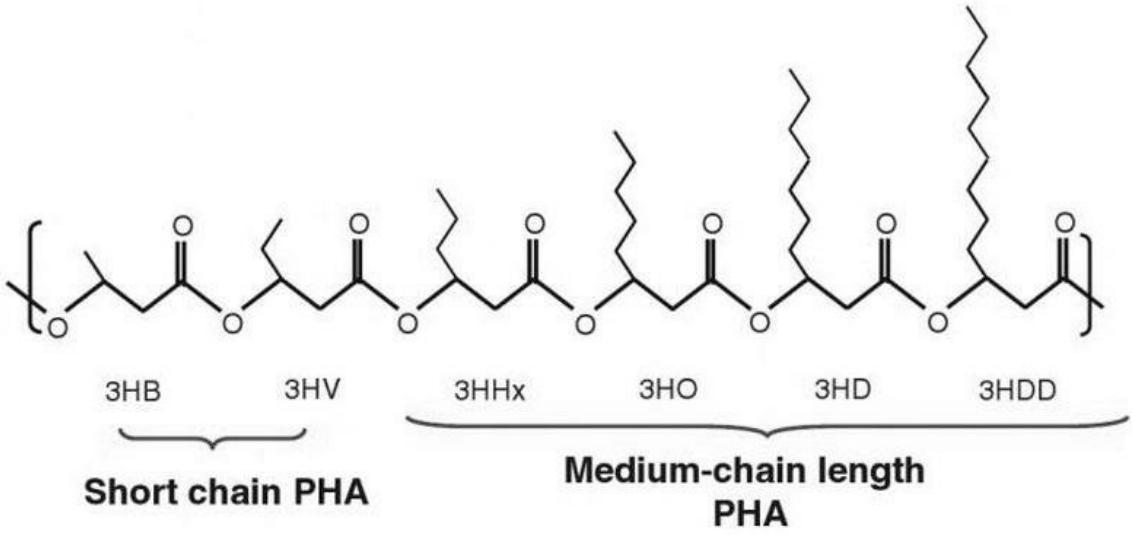

Figure 2.5: Common PHA monomer structures. Short-chain-length monomers: 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), Medium-chain length monomers: 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDD) [46].

As mentioned before, PHAs are reported to be biosynthetic, biodegradable and potentially biocompatible polymers with interesting properties similar to conventional petroleum derived plastics [22-24]. Formation of PHA granules is dependent on the presence of suitable metabolic pathways. There are four different pathways for the synthesis of PHAs found to date. These

pathways in detail have been reported elsewhere [53, 54]. Figure 2.6 shows a simplified metabolic pathway for the biosynthesis of poly(3-hydroxybutyrate) P(3HB), which is the most common type of PHA, in *Ralstonia eutropha*, The biosynthesis of P(3HB) is initiated by the condensation of two acetyl-CoA molecules by β-ketothiolase (PhaA) to form acetoacetyl-CoA. An NADPH-dependent acetoacetyl-CoA reductase then carries out its conversion to 3-hydroxybutyryl-CoA which is then polymerized in P(3HB) by PHA synthase [55, 56].

According to Charles Forrester [57], when normal growth of the biomass is prevented by nutrient limitation, e.g. relatively high C/N ratios, the citrate synthase is inhibited, preventing acetyl-CoA (a precursor for PHB synthesis) from entering the citric cycle and allowing for PHB accumulation.

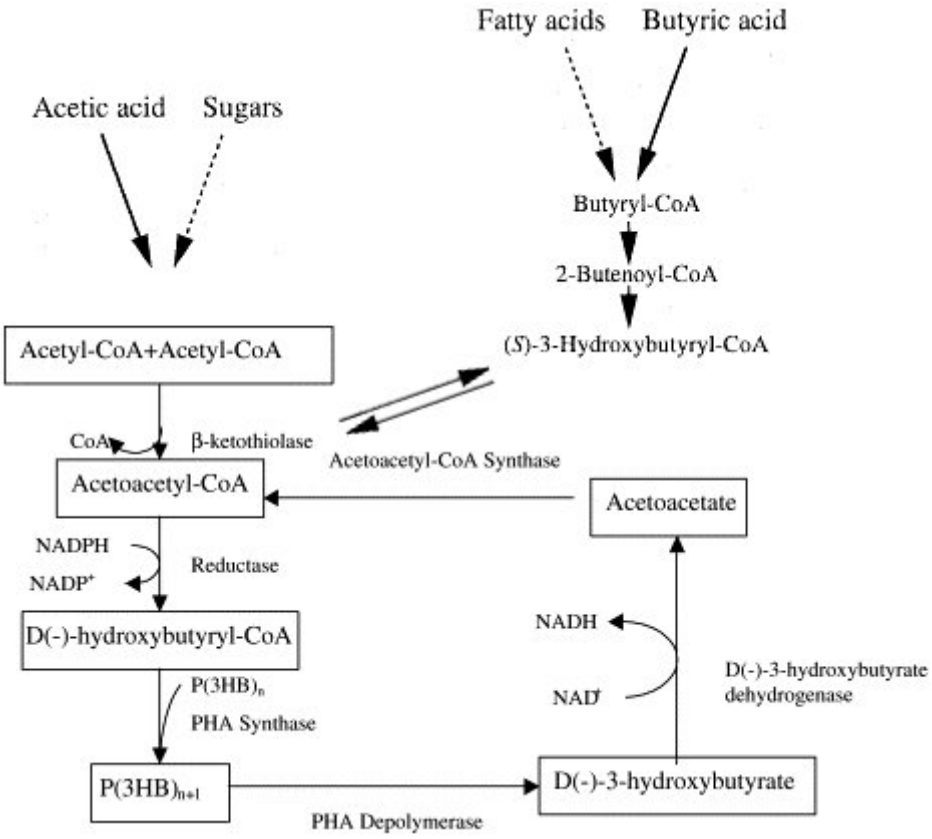

Figure 2.6: Metabolic pathway involved in the synthesis and breakdown of PHB in Ralstonia eutropha (adapted from [55, 56]).

2.5.1. POLYHYDROXYBUTYRATE

P(3HB) was the first PHA discovered in *Bacillus megaterium* by the French scientist Lemoigne in 1926 [58]. He reported this bacterium to accumulate intracellularly a homopolymer that consisted of 3-hydroxybutyric acids that were linked through ester bonds between the 3-hydroxyl group and the carboxylic group of the next monomer [59].

The length of the side chain of PHAs and its functional group considerably influence the properties of the bioplastics, e.g., melting point, glass transition temperature, and crystallinity (stiffness/flexibility) [60]. Also the average molecular weight and the molecular weight distribution [61] are dependent on the carbon source [62]. Specifically, PHB is a highly crystalline thermoplastic polymer with a relatively high melting temperature (T_m) (175-177°C), a glass transition temperature (T_g) of 4°C and crystallinity around 80% [63, 64]. PHB is water insoluble and relatively resistant to hydrolytic degradation. This differentiates PHB from most other currently available biodegradable plastics, which are either water soluble or moisture sensitive. Moreover, it has some particular properties such as: good oxygen permeability, good ultraviolet (UV) resistance but poor resistance to acids and bases, soluble in chloroform and other chlorinated hydrocarbons, biocompatible and hence suitable for medical applications, tensile strength of 40 MPa, which is near to common plastics (propylene), sinks in water (while propylene floats), facilitating its anaerobic biodegradation in sediments and non-toxic [64].

2.5.2. MICROBIAL CULTURES

Storage polymers are synthesized by numerous microorganisms such as Gram-negative bacteria, Gram-positive bacteria, aerobic (cyanobacteria) and anaerobic bacteria, as well as in some archaebacteria [51, 56, 65]. Therefore, PHAs can be produced by pure cultures or by mixed cultures. Both offer some advantages and disadvantages regarding each other.

2.5.2.1. Pure cultures

All the industrial processes implemented so far use pure microbial cultures that can be wild or genetically modified strains [66-68]. The main cost drivers of the process are related to fermentation (energy for sterilization and substrate cost) and downstream processing (a substantial number of unitary operations, chemicals and energy demand) [69]. Substrates used in the established industrial processes are, in almost all the cases, pure sugars, such as glucose or sucrose, which have a high market price [67, 70, 71]. Only well-defined nutrient-deficient substrate mixtures can be used in pure cultures as the storage is induced by nutrient deficiency. The few advantages of using pure cultures are that it is possible to obtain high intracellular polymer concentrations, the pure cultures do not require the use of a feast and famine regime and produce specific PHAs, both through natural on recombinant strains. Table 2.1 gives an overview of some studies in PHB production using pure cultures.

Table 2.1: Overview of PHB production and yield in pure cultures (adapted from [72])

Strain	Carbon source	PHB content (% on CDW)	Yield (g PHB/g substrate)	References
Alcaligenes eutropha	Glucose	76	0.30	Choi and Lee [69]
Alcaligenes latus	Sucrose	88	0.42	Choi *et al.* [73]
Methylobacterium organophilum	Methanol	52	0.19	Choi *et al.* [73]
Recombinant *Escherichia coli*	Glucose	77	0.27	Choi *et al.* [73]

CDW: Cell Dry Weight

2.5.2.2. Mixed cultures

Many different approaches based on the use of mixed cultures processes have been proposed, but none have yet been implemented at industrial scale [74]. Synthesis of PHA by mixed cultures was first observed in wastewater treatment plants (WWTP) designed for enhanced biological phosphorus removal (EBPR) [75]. The capability to produce and store PHA is selected by submitting the sludge to dynamic feeding conditions, such as aerobic dynamic or feast and famine regime, through altering between high and low/none carbon substrate concentration [76]. These dynamic feeding conditions are applied because not all the strains present in these mixed cultures are capable to store polymers. Therefore, it is possible to enrich the mixed cultures in microorganisms capable to store PHA.

PHAs synthesis by mixed cultures can decrease the production costs, since during production sterilization of equipment and control requirements are not necessary and the microbial communities in activated sludge can adapt well to the complex substrates present in the agro industrial wastes. Also, there is generation of wealth from waste and viable environment-friendly processes [77, 78].

Production of PHA by mixed microbial cultures and wastes as substrate seems to have many advantages when compared to the already well-known process where pure cultures and single substrates are used. However, in spite of the many efforts devoted in the last decade to improve the process, one problem has yet to be solved: the lower PHA content and volumetric productivities than those achieved for pure cultures [74].

Because the mixed microbial culture (MMC) process is based on the use of open cultures, the possibility of using the facilities already existing in wastewater treatment plants to produce PHA should be explored as a cost-effective technology. The implementation of this process still

requires some research, namely on the quality and stability of the produced polymer. In this respect, it is mandatory to find simple polymer recovery processes and applications for PHA where less purity of the polymer is required [74]. Thus, this process can be economically competitive with PHA production from pure cultures and it has the advantages of being simpler and requiring less investment and operating costs [79-83].

Table 2.2: Overview of PHB production and yield in mixed cultures (adapted from [72]).

Culture	Carbon source	PHB content (% on CDW)	Yield (g PHB/g substrate)	References
Mixed culture	Excess sugar	65 – 70	–	Castilho *et al.* [84]
	Acetate	Up to 84	–	Johnson, K. *et al.* [85]
Enrichment culture	Acetic, lactic and propionic acid	50	0.39 (substrate as COD)	Dionisi, D. *et al.* [79]
Activated sludge	Effluent anaerobically fermented sludge	Up to 57	–	Cai, M.M. *et al.* [78]
	Municipal wastewater	21	–	Chua, A.S.M. *et al.* [86]
Sewage bacteria	Food waste	Up to 51	0.05 (substrate as COD)	Rhu, D.H. *et al.* [87]

CDW – Cell Dry Weight

2.5.3. PHA APPLICATIONS

Notwithstanding many environmental concerns on pollution caused by petroleum-based plastics, research on bioplastics has been developed slowly during the past few years. As a consequence of the ready biodegradability of PHAs in soils and domestic waste treatment systems [88], PHB and its copolymers were already used in many applications, mostly replacing the conventional petroleum derived plastics, as can be verified in Table 2.3.

PHAs have also been studied for biocompatibility as various biomedical applications which could also imply potential applications for polydydroxyalkanoate-diethylene glycol (PHA-DEG) hybrids biomaterials. PHB and poly[(R)-3-hydroxybutyrate-*co*-(R)-3-hydroxyvalerate] (PHBV) have, in particular, been studied extensively as potential drug delivery systems and scaffolds in tissue engineering. [47, 59, 89]. Table 2.3 gives more examples where PHA has been applied.

Table 2.3: Applications of PHA in various fields (adapted from [90])

Applications	Examples
Packaging industry	All packaging materials that are used for a short period of time, including food utensils, films, daily consumables, electronic appliances etc
Printing & photographic industry	PHA are polyesters that can be easily stained
Other bulk chemicals	Heat sensitive adhesives, latex, and smart gels. PHA nonwoven matrices can be used to remove facial oils
Block copolymerization	PHA can be changed into PHA diols for block copolymerization with other polymers
Plastic processing	PHA can be used as processing aids for plastic processing
Textile industry	Like nylons, PHA can be processed into fibers
Fine chemical industry	PHA monomers are all chiral R-forms, and can be used as chiral starting materials for the synthesis of antibiotics and other fine chemicals [91]
Medical implant biomaterials	PHA have biodegradability and biocompatibility, and can be developed into medical implant materials [92]. PHA can also be turned into drug controlled release matrices
Medical	PHA monomers, especially R3HB, have therapeutic effects on Alzheimer's and Parkinson's diseases, osteoporosis and even memory improvement [93-95]
Healthy food additives	PHA oligomers can be used as food supplements for obtaining ketone bodies [96]
Industrial Microbiology	The PHA synthesis operon can be used as a metabolic regulator or resistance enhancer to improve the performances of industrial microbial strains [97, 98]
Biofules or fuel additives	PHA can be hydrolyzed to form hydroxyalkanoate methyl esters that are combustible [99]
Protein purification	PhaP are used to purify recombinant proteins [100]
Specific drug delivery	Co-expression of PhaP and specific ligands can help achieve specific targeting to diseased tissues

PhaP: PHA granule binding phasin; R3HB: (R)-3-hydroxybutyric acid

2.5.4. Approaches to PHA production

2.5.4.1. Mechanisms of feeding

In recent years many studies refer the production of PHAs by activated sludge when exposed to transient carbon supply [101, 102]. Under these dynamic conditions, sludge submitted to consecutive periods of external substrate accessibility (''feast'') and unavailability (''famine'') generates a so-called unbalanced growth. During the excess of external carbon substrate, the uptake is mainly driven to PHA storage and, to a lesser extent, to biomass growth. After substrate exhaustion, the stored polymer can be used as an energy and carbon source. [103]. Effectively, the polymer acts as a buffer for the substrate that is taken up but not directly used for growth. In this way, growth of biomass can continue at a similar or slightly decreased rate in periods without

external substrate supply. The whole mechanism enables the bacteria to maintain their growth at a more or less constant, or balanced, rate and efficiently compete for substrate under dynamic substrate supply [101]. The ability to store internal reserves gives these microorganisms a competitive advantage over those that do not have this capacity, thereby becoming dominant in systems submitted to transient conditions [102].

2.5.4.2. Growth conditions

2.5.4.2.1. pH

A few published studies have described the effect of pH on the rate and yield of polymer accumulation, typically indicating a higher polymer production when the pH is controlled in the range from 8 to 9. Furthermore, a higher PHA production rate in the stored polymer was observed at alkaline pH values in the range from 7.5 to 9.0 [86, 103-105].

2.5.4.2.2. Oxygen

The dissolved oxygen (DO) is one determinant factor in the systems with feast and famine regime. It seems to be important to maintain oxygen saturation in the mixed liquor of at least 2.0 mg/L [106, 107]. In the beginning of the feast phase, the oxygen uptake decreases immediately when the substrate is added. This means that the microbial biomass is metabolizing the substrate. Afterwards, the concentration of dissolved oxygen remains constant during the feast phase and then starts to increase due to the growth of the biomass [79].

2.6. PHA RECOVERY METHODS

Several technologies to recover the PHA from the biomass have been proposed and studied on small scale in the laboratory as well as at industrial scale. These include solvent extraction, chemical digestion, enzymatic treatment and mechanical disruption.

2.6.1. SOLVENT EXTRACTION

Solvent extraction is the most extensively used technique to recover PHA from the cell biomass (Table 2.4). This method is also used routinely at lab-scale because of its simplicity and rapidity. Two main steps are involved, first is the modification of cell membrane permeability thus allowing release and solubilization of PHA, followed by non-solvent precipitation [108]. Chlorinated hydrocarbons are the common solvents used in extraction of PHA, i.e. chloroform,

1,2-dichloroethane [109] or some cyclic carbonates like ethylene carbonate and 1,2-propylene carbonate [110, 111]. Precipitation of PHA, the second step of the solvent extraction, is commonly induced by non-solvent such as methanol and ethanol [109].

Solvent extraction has undoubtedly advantages over other extraction techniques of PHA in terms of efficiency. Furthermore, it is also able to remove bacterial endotoxin and causes negligible degradation to the polymers [108]. Therefore, pure PHA with high molecular weights is obtained by solvent extraction. At a large scale, solvent extraction is generally considered as a non-environmentally friendly method. In case of accidents, the potential release of a large amount of highly toxic and volatile solvents to the environment is a major concern [108, 112, 113]. Moreover, several other factors discourage the use of solvents such as high capital and operational costs. Another problem is the high viscosity of the extracted polymer solution when the PHB concentration exceeds 5% (w/v) (Table 2.4). Therefore, 1,2-propylene carbonate has been proposed as an alternative to halogenated solvents in the recovery process of PHA [114, 115].

Table 2.4: Reported applications of solvent extraction as a PHA recovery method [110]

Extraction method	Comments	Strain	Results	Reference
Solvent extraction	Chloroform	*Bacillus cereus* SPV	Purity: 92% Yield: 31%	Valappil *et al.* [116]
	Chloroform	*Cupriavidus necator* DSM 545	Purity: 95% Yield: 96%	Fiorese *et al.* [115]
	1,2-Propylene carbonate	*Cupriviadus necator* DSM 545	Purity: 84% Yield: 95%	Fiorese *et al.* [115]
	Acetone-water process		Yield: 80-85%	Narasimhan *et al.* [117]
	Methyl *tert*-butyl ether	*Pseudomonas putida* KT2440	Yield: 15–17.5 wt%	Wampfler *et al.* [118]
	Methylene chloride	*Cupriviadus necator*	Purity: 98%	Zinn *et al.* [59]
	Non halogenated solventsisoamy propionate, propyl butyrate, isoamyl valerat etc	*Cupriviatus necator*	-	Mantelatto and Durao [119]
	Acetone, room temperature	*Pseudomonas putida* GPo1	Yield: 94%	Elbahloul and Steinbüchel [120]

Higher boiling point (240°C) of 1,2-propylene carbonate prevents the evaporation to the environment at lower temperatures and allows its reusability for several cycles of purification. This could decrease the solvent consumption and therefore it is viewed as an economically advantage. This solvent is widely used in many applications including cosmetics [114]. Fiorese *et al.* [115] reported a maximum PHA yield of 95% and a purity of 84% when extracted from the *Cupriavidus necator* cells at 130°C for 30 min without involving any pretreatment. Despite chloroform extraction values are slightly better, 96% yield and 95% purity, the results using 1,2-propylene carbonate are promising.

2.6.2. DIGESTION METHODS

While solvent extraction techniques involve the solubilization of the PHA granules, digestion methods involve the solubilization of the cellular materials surrounding the PHA granules. The digestion methods can be divided in two main groups: the chemical digestion and the enzymatic digestion.

2.6.2.1. Chemical digestion

Several chemical digestions have been evaluated for the recovery of PHA from cellular biomass (Table 2.5). The main chemical compounds used in this technique are surfactants or sodium hypochlorite. These compounds are used because they can solubilize the non-PHA cellular mass, facilitating PHA recovery. A range of surfactants has been evaluated such as sodium dodecyl sulfate (SDS), Triton X-100, palmitocyl carnitine and betaine, and among them SDS showed good performance. However, the quality of PHA obtained using either surfactant or sodium hypochlorite alone was not good enough (Table 2.5). Therefore, a combination of surfactant and sodium hypochlorite was used [113]. Sequential surfactant-hypochlorite treatment promoted better and rapid recovery of PHA [121, 122] and resulted in 50% reduction of overall cost when compared to solvent extraction [112]. On the other hand, the low operating cost [108] and technical simplicity of this process are not complemented by some problems caused by surfactant in wastewater treatment and relatively high cost of chemical agents such as SDS and sodium hypochlorite [112]. Table 2.5 contains some results concerning yields and purity of PHB that have been reported for this method.

2.6.2.2. Enzymatic digestion

Recovery process of PHA using enzymatic digestion involves a rather complex procedure. Solubilization of cell components, except PHA, typically consists of heat treatment, enzymatic hydrolysis and surfactant washing [123]. To date, several types of enzymes, especially proteases, have been evaluated for their efficiency in causing cell lysis [124]. Some studies have been reported which used microorganisms to secrete protease and subsequently reduce the cost of this method [125, 126].

This technique is attractive because enzymes are very specific in the reactions that they catalyze, are environmentally friendly of its operation conditions [127] and the recovery of PHA with good quality could be expected (Table 2.5) [127, 128]. Nevertheless, the high cost of enzymes and complexity of the recovery process outweigh its advantages [128].

Table 2.5: Reported applications of digestion methods for PHA recovery [110]

Digestion methods				
Extraction method	**Comments**	**Strain**	**Results**	**Reference**
Surfactant	SDS	Recombinant *Escherichia coli*	Purity: 99% Yield: 89%	Choi and Lee [129]
	Palmitoyl carnitine	*Cupriavidus necator*, *Alcaligenes latus*	Degree of lysis: 56-78%	Lee *et al.* [130]
Sodium hypochlorite	Sodium hypochlorite	*Cupriavidus necator*	Purity: 86%	Hahn *et al.* [131]
		Recombinant *E. coli*	Purity: 93%	
	Sodium hypochlorite	*C. necator* DSM 545	Purity: 98%	Berger *et al.* [132]
Surfactant-sodium hypochlorite	SDS-Sodium hypochlorite	*Azotobacter chroococcum* G-3	Purity: 98% Yield: 87%	Dong and Sun [122]
	Triton X-100-sodium hypochlorite	*Cupriavidus necator* DSM 545	Purity: 98%	Ramsay *et al.* [121]
Surfactant-Chelate	Triton X-100-EDTA	*Sinorhizobium meliloti*	Purity: 68%	Lakshman and Shamala [125]
	Betaine-EDTA disodium salt	*Cupriavidus necator* DSM 545	Purity > 96% Yield: 90%	Chen *et al.* [133]
Dispersion of sodium hypochlorite and chloroform	Chloroform- sodium hypochlorite	*Bacillus cereus* SPV	Purity: 95% Yield: 30%	Valappil *et al.* [116]
	Chloroform- sodium hypochlorite	*Cupriviadus necator*, Recombinant *E. coli*	Purity: >#98%	Hahn *et al.* [131]
Enzymatic digestion	*Microbispora* sp culture-chloroform	*Sinorhizobium meliloti*	Purity: 94%	Lakshman and Shamala [125]
	Enzyme combined with SDS-EDTA	*Pseudomonas putida*	Purity: 93%	Kathiraser *et al.* [134]
	Bromelain	*Cupriavidus necator*	Purity: 89%	Kapritchkoff *et al.* [128]

2.6.3. MECHANICAL DISRUPTION

Mechanical cell disruption is widely used to liberate intracellular protein [135]. The concept has been tested to recover PHA from bacterial cells [113]. Among the several mechanical disruption methods, high-pressure homogenization and bead milling dominate the large scale cell disruption in pharmaceutical and biotechnology industries [136]. In contrast with other recovery methods, mechanical disruption is chosen mainly due to economic advantage and because it causes insignificant damage to the products [113]. Mechanical disruption of cells does not involve any chemicals so it minimizes environmental pollution [108] and contamination to the products [113]. In general, the drawbacks of mechanical disruption method are, high capital investment cost, long processing time and difficulty in scaling up [137, 138].

2.6.3.1. High pressure homogenization

With high pressure homogenization, disruption of cell suspension occurs under high pressure through an adjustable, restricted orifice discharge valve [139]. Process parameters such as operating pressure, number of steps, suspension temperature and homogenizer valve design, must be carefully analyzed for efficient disruption [140, 141]. Not only process parameters influence the cell disruption, also microbial physiological parameters, namely type and growth phase of the microorganisms, as well as cell concentration, affect the disruption efficiency (Table 2.6). Generally, Gram-positive bacteria are more difficult to be disrupted compared to Gram-negative bacteria [141]. Drawbacks associated with high pressure homogenization include the possibility of thermal degradation of desired products [142] and formation of fine cellular debris that would interfere with the further downstream processing of PHA granules [140].

2.6.3.2. Beads mill

The principle of beads mill is based on the shearing action and energy transfer from beads to cells in the contact zones. The key parameters that affect the disruption process are the bead loading and bead diameter [127].

Beads mill disruption was recommended for PHA recovery as it requires less power supply, not susceptible to blockages, and different diameters of beads did not significantly affect the disruption rate [113]. The major concern is that a large number of factors has to be considered to establish a good beads mill disruption system (Table 2.6) [143].

Table 2.6: Reported applications of mechanical disruption as a PHA recovery method

Extraction method	Comments	Strain	Results	Reference
Mechanical disruption	Bead mill	*Alcaligenes latus*	-	Tamer *et al.* [113]
	High pressure homogenization	*Alcaligenes latus*	-	Tamer *et al.* [113]
	SDS-High pressure homogenization	*Metylobacterium* sp V49	Purity: 95%; Yield: 98%	Ghatnekar *et al.* [142]
	Sonication	Baci*llus flexus*	Purity: 92%; Yield: 20%	Divyashree *et al.* [126]

Overall, the enzymatic digestion seems to be more environmentally friendly. However, pure enzymes are costly. In order to reduce the cost, some researchers have used whole microbial cultures as the source of the enzymes. It has been reported that the use of *Microbispora* sp. culture instead of pure enzymes to hydrolyze *Sinorhizobium meliloti* cells can be a promising procedure [125, 126]. Some methods like bead mill, high pressure homogenization and supercritical fluid disruption are also environmentally friendly since no chemicals are involved in the PHA recovery process. Table 2.7 summarizes the balance of the advantages and disadvantages of the several PHA extraction methods mentioned previously.

Table 2.7: Comparison of the advantages and disadvantages of various PHA extraction methods [110]

Recovery method	Advantages	Disadvantages
Solvent extraction	Removal of endotoxin Useful for medical applications High purity Negligible/limited degradation to the polymer Higher molecular weight	Not environmentally friendly Consumption of large volume of toxic and volatile solvents High capital and operation cost Difficulty in extracting PHA from solution containing more than 5% (w/v) PHB Lengthy process Native order of polymer chains in PHA granules might be disrupted
Chemical Digestion Surfactant	Extracted PHA retains original molecular weight Native order of polymer chains in PHA granules is retained	Low purity of PHA Treatment required to remove surfactant from wastewater
Sodium hypochlorite	Higher purity of PHA can be obtained	Severe reduction in molecular weight of the extracted PHA
Sequential surfactant hypochlorite	High quality of PHA Rapid recovery and simple process Retain native order of polymer chains in PHA granules Lower operating cost compared to solvent extraction	Combined cost of surfactant and sodium hypochlorite Wastewater treatment required to remove residual surfactant and sodium hypochlorite

Table 2.7: Continued

Recovery method	Advantages	Disadvantages
Dispersion sodium hypochlorite and solvent extraction	High purity of PHA Reduced viscosity of solvent phase due to digestion of non-polymer cellular material by sodium hypochlorite	Not environmentally friendly Consumption of large volume of toxic and volatile solvents Higher recovery cost
Surfactant-chelate	Convenient operation High quality of product	Produce large volume of wastewater
Enzymatic digestion	Mild operation conditions Good recovery with good quality	Complex process High cost of enzymes
Bead mill	No chemicals used Less contamination Not susceptible to blockages No micronization of PHA granules	Require several passes Long processing time Various process parameters have to be controlled precisely
High pressure homogenization	No chemicals used Less contamination	Severe micronization of PHA granules Depends on both process and microbial physiological parameters Possible for thermal degradation of desired products Formation of fine cellular debris that interfere with downstream processing

2.7. LARGE SCALE PRODUCTION OF PHA

Generally, PHA production contains several steps, including fermentation, separation of biomass from the medium, biomass drying, PHA extraction, PHA drying, and packaging. A scheme of the whole PHA production process can be observed in Figure 2.7, including all those steps mentioned before.

Through the years, several companies have invested in PHA research & development and production. Globally, there are 24 companies known to have engaged in PHA research & development as well as production (Table 2.8) [90]. Some of them have stopped their PHA activities, mainly in the 1990s, owing to the low petroleum price. PHA has experienced another increase in attention since the oil price increased to over US $ 100 per barrel in early 2003 [46].

Although many PHAs have been found, only four of them have been produced on a large scale for commercial exploration. These are (P3HB), PHBV, poly[(R)-3-hydroxybutyrate-*co*-4-hydroxybutyrate] (P3HB4HB), and poly[(R)-3-hydroxybutyrate-*co*-(R)-3-hexanoate] (PHBHHx) [46].

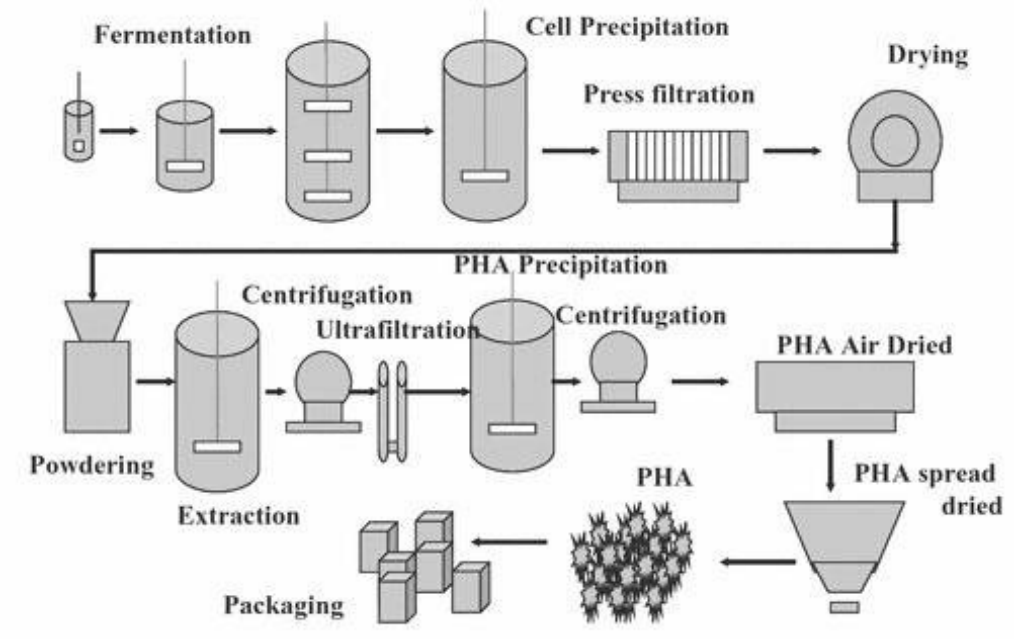

Figure 2.7: General polyhydroxyalkanoate production and extraction process [46].

One of the strains that meets the requirements for industrial production of PHB is *Alcaligenes latus* [144, 145]. The strain grows quickly in sucrose, glucose and molasses. The accumulation of PHB can reach more than 90% of their cell dry weight (CDW). There was even been an attempt to produce PHB from waste materials using *A. latus* [144]. Chemie Linz, Austria (later btf Austria) produced PHB in an amount of 1,000 kg per week in a 15-m^3 fermentor using *A. latus* DSM 1124 [63]. The cells were grown in a mineral medium, containing sucrose as a carbon source. The PHB produced by *A. latus* was subsequently used to make sample cups, bottles and syringes for application trials. Production and processing technology of PHB are now owned by Biomer, Germany. So far, different products, including combs, pens and bullets, have been made due to PHB produced by *A. latus* [46].

Table 2.8: Worldwide PHA producing and researching companies [90]

Company	Types of PHA	Production scale (t/a)	Period	Applications
ICI, UK	PHBV	300	1980s to 1990s	Packaging
Chemie Linz, Austria	PHB	20-100	1980s	Packaging & drug delivery
btF, Austria	PHB	20-100	1990s	Packaging & drug delivery
Biomers, Germany	PHB	Unknown	1990s to present	Packaging & drug delivery
BASF, Germany	PHB	Pilot scale	1980s to 2005	Blending with Ecoflex

Table 2.8: Continued

Company	Types of PHA	Production scale (t/a)	Period	Applications
Metabolix, USA	Several PHA	Unknown	1980s to present	Packaging
Tepha, USA	Several PHA	PHA medical implants	1990s to present	Medical bio-implants
ADM, USA (with Metabolix)	Several PHA	50 000	2005 to present	Raw materials
P&G, USA	Several PHA	Contract manufacture	1980s to 2005	Packaging
Monsanto, USA	PHB, PHBV	Plant PHA production	1990s	Raw materials
Meredian, USA	Several PHA	10 000	2007 to present	Raw materials
Kaneka, Japan (with P&G)	Several PHA	Unknown	1990s to present	Packaging
Mitsubishi, Japan	PHB	10	1990s	Packaging
Biocycles, Brazil	PHB	100	1990s to present	Raw materials
Bio-On, Italy	PHA (unclear)	10 000	2008 to present	Raw materials
Zhejiang Tian An, China	PHBV	2000	1990s to present	Raw materials
Jiangmen Biotech Ctr, China	PHBHHx	Unknown	1990s	Raw materials
Yikeman, Shandong, China	PHA (unclear)	3000	2008 to present	Raw materials
Tianjin Northern Food, China	PHB	Pilot scale	1990s	Raw materials
Shantou Lianyi Biotech, China	Several PHA	Pilot scale	1990s to 2005	Packaging and medical
Jiang Su Nan Tian, China	PHB	Pilot scale	1990s to present	Raw materials
Shenzhen O'Bioer, China	Several PHA	Unknown	2004 to present	Unclear
Tianjin Green Bio-Science (+DSM)	P3HB4HB	10 000	2004 to present	Raw materials & packaging
Shandong Lukang, China	Several PHA	Pilot scale	2005 to present	Raw materials & medical

Ralstonia eutropha is capable of growing on glucose and producing the copolymer Poly(3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) to a density as high as 70-80 g/L after over 70 h of growth [146]. Shampoo bottles have been made from PHBV (trademarked as Biopol) and have been available in supermarkets in Europe. However, due to economic reasons, the Biopol products did not succeed and the PHBV patents were sold to Monsanto and further after to Metabolix [46, 147].

NingBo TianAn, China, in collaboration with the Institute of Microbiology affiliated with the Chinese Academy of Sciences, has developed a model process that can is capable of producing PHBV in high efficiency. Without any supply of pure oxygen, *Ralstonia eutropha* grew to a density of 160 g/L CDW within 48 h in a 1000 L fermenter. The cells could accumulate PHBV to an amount of 80% of their constitution with a production efficiency of 2.5 g/h.L. The hydroxyvalerate content in the copolymer ranged from 8 to 10%. This process could significantly diminish the production cost for PHBV. Only by achieving a high growth rate, a high PHBV production efficiency, and high cell and PHBV densities, the polymers can become economically competitive. We assume that PHBV or other PHAs will turn out to be cost-effective after extensive improvement of the fermentation process and the downstream process [46, 147].

Ralstonia eutropha and recombinant *Escherichia coli* are used by Tianjin Green Bioscience, China, and Metabolix, USA, to produce P3HB4HB. By adding different amounts of 1,5-batanediol, 4-hydroxybutyrate can be accumulated to 5-40 mol % in the copolymer, to subsequently obtain copolymers with various thermal and mechanical properties for various applications. Tianjin Green Bioscience and Metabolix are building facilities with capacities of 10 000 and 50 000 tons of P3HB4HB, respectively. Then, P3HB4HB will be the PHA available on the market in the greatest quantity. At the same time, both companies are working on the development of various bulk applications [46].

The PHBHHx production was performed on glucose and lauric acid for about 60 h. The CDW reached was 50 g/L and 50% of PHBHHx was accumulated by the biomass. The production cost for PHBHHx is currently still too high for real commercial application. However, much energy has been put in the attempt to improve the production process for PHBHHx, including the downstream process technology. Most efforts focused on increasing the cell density and simplifying the downstream process. One of the most important issues to decrease the PHBHHx production costs will be a better production strain able to utilize glucose [148].

Akiyama *et al*. [149] simulated large-scale fermentative production of PHBHHx with 5 mol% (R)-3-hydroxyhexanoate (HHx) [P(3HB-*co*-5 mol% 3HHx)] from soybean oil as the only carbon source using a recombinant strain of *Wautersia eutropha* harboring a PHA synthase gene from *Aeromonas caviae*. The costs of the annual production of 5,000 tons of P(3HB-*co*-5 mol% 3HHx) are estimated from US \$3.5 to 4.5 kg^{-1}, depending on the expected production performances. Similar-scale production of PHB from glucose is estimated to cost US \$ 3.8-4.2/5 kg of PHB [46].

2.7.1. PROSPECTS

Looking at the companies that are still in the PHA business, it is possible to observe that a representative part has finished their activity. Recent news revealed that the collaboration between ADM and Metabolix ended. Those companies were most likely the major players in the PHA market. Lack of results and huge losses were responsible for this termination. This indicates that there must be found a different way to produce PHAs.

Nowadays PHAs are mainly produced as raw and packaging material. However in this market it competes directly with the much cheaper synthetic polymers or with more established bioplastics as PLA. While synthetic plastics cost around 1.0 €/kg and PLA cost between 2.5 to 5€/kg, the price of PHAs ranges between 6.6 to 10.0€/kg [150]. This price difference has been a major barrier to the acceptance on large scale of PHAs as a viable alternative to the synthetic plastics.

To reach the economic success of PHA a different approach is required. One possible approach is to find a field of application for them, where they could outstand from the competition and their cost be overlapped by their benefits. Another approach is to implement new production and recovery technologies in order to decrease their cost, as the price of PHA depends on the substrate cost, energy demand, PHA yield, and on the downstream process of the polymer [70].

CHAPTER 3

PRODUCTION OF POLYHYDROXYALKANOATES FROM A SYNTHETIC MEDIUM BY (OPEN) MIXED CULTURES AT PILOT SCALE

3.1. SUMMARY AND MOTIVATION

For several years, the interest in sustainable means of production, as well as the shift of raw materials base from fossil to renewable resources is gaining importance in biotechnological industries. Modern biotechnology provides groundbreaking products and technologies to combat debilitating and rare diseases, reduce our environmental footprint, feed the hungry, use less and cleaner energy, and have safer, cleaner and more efficient industrial manufacturing processes.

The study described in this chapter was performed at Avecom NV in Gent, Belgium. Avecom NV is a company that focuses on the development of efficient products for numerous applications, such as aerobic wastewater treatment, anaerobic wastewater treatment, aquaria and ponds, soil and groundwater remediation. Avecom NV has also experience in sludge analysis, biodegradation, nitrification/denitrification and eco-toxicity tests, providing also advice regarding, mainly, wastewater treatments.

The proposed challenge was to produce 25 kg of a novel product, biomass enriched in polyhydroxyalkanoates (PHAs), to be afterwards subjected to some tests in view of a possible application. To accomplish the challenge, a pilot reactor (600L) was operated in a feast and famine regime to select organisms that easily store intracellular carbon reserves during a feast phase, which can then be used as energy source during a famine phase. In a second step, the biomass of the pilot reactor was harvested and filled up with PHAs. During the fill-up phase, the biomass was supplied with acetic acid, but nutrients were deficient, in order to maximize the storage of carbon reserves. In a third step, the filled-up biomass was stabilized by addition of calcium hydroxide ($Ca(OH)_2$). The pH shift was necessary to make sure that bacteria died (hygienization) and to prevent conversion of the product (PHA). The forth step consisted in thickening and drying the biomass by physical techniques. Finally, the final product was pulverized and stored at room temperature.

3.2. MATERIALS AND METHODS

3.2.1. INOCULUM AND SUBSTRATES

3.2.1.1. Inoculum

The inoculum used in the pilot scale Sequencing Batch Reactor (SBR) – selector reactor - was an activated sludge obtained from a SBR wastewater treatment of a brewery in Ghent, Belgium.

3.2.1.2. Substrates

The substrate composition may have a direct influence on the microbial community structure of the biomass, which will subsequently affect the PHA producing capacity.

In these experiments, a synthetic media was used as substrate. The synthetic medium 1 contained acetic acid (CH_3COOH) as a carbon source, urea ($(NH_4)_2CO$) and soy peptone as a nitrogen source, dipotassium phosphate (K_2HPO_4) as a phosphate source, magnesium sulphate ($MgSO_4$) and a trace element, Nutriflok®, from Avecom NV in Gent, Belgium. The final composition of each element is described in Table 3.1. The synthetic medium 2 was a solution of acetic acid (CH_3COOH) with 40 g/L.

Table 3.1: Composition of the synthetic media used in the experiments

Compound	Unit	Synthetic medium 1	Synthetic medium 2
(CH_3COOH) (80%)	mL/L	12.5	50
$((NH_4)_2CO)$	mL/L	2.1	-
Soy peptone	g/L	0.5	-
K_2HPO_4	g/L	2.292	-
Nutriflok®	g/L	0.10	-
$MgSO_4 7H_2O$	g/L	0.179	-
COD	g/L	10.0	40,0
$((NH_4)_2CO)$ – N	g N/L	0.435	-
K_2HPO_4 - P	g P/L	0.408	-
COD/N/P	-	100/4.35/4.08	-

COD: Chemical Oxygen Demand

3.2.2. ANALYTICAL METHODS

3.2.2.1. Total solids (TS), lipid content, volatile solids (VS), total suspended solids (TSS) and volatile suspended solids (VSS) contents

The total solids (or dry matter - DM) content in the samples was determined by the dry residue method and the volatile solids content by the ash content method. The dry residue is defined as the residue remaining after evaporation of the water present in the sample volume at 105°C, while the ash content is defined as the residue remaining after incineration of the dry residue at 600°C [151]. As in volatile solids, the lipid content was measured by incineration of the dry residue, but instead of using a temperature of 600°C, a temperature of 250°C was used [152].

Following this method, a certain amount of sample was placed in a dry porcelain crucible, previously weighed by means of a Sartorius TE64 Analytic Balance. Then, the crucible was introduced in the oven from Memmert overnight at 105°C. After cooling down, the crucible was weighed and the TS content was given by the difference between the porcelain crucible with the dry residue and the porcelain crucible as such divided by the volume of the sample. Subsequently, the porcelain crucible with the dry matter was placed in the incinerator Nabertherm GmbH LE 4/11/R6 at 250°C for 2 hours. After cooling down in a desiccator, the porcelain crucible was weighed and the lipid content was given by the difference between the weight of dry solids (105°C) and the total solids remaining after 250°C, divided by the sample volume. Subsequently, the porcelain crucible with the dry residue was placed in the incinerator Nabertherm GmbH LE 4/11/R6 at 600°C for 2 hours. After cooling down in a desiccator, the porcelain crucible was weighed and the ash content was given by the difference between the porcelain crucible with the ashes and the porcelain crucible as such, divided by the sample volume. Finally, the VS content was given by subtracting the ash content from the TS content previously determined.

Total suspended solids and volatile suspended solids measurement followed the same method as total and volatile solids, respectively, but the sample was previously centrifuged at 10000 rpm for 10 min (Eppendorf Centrifuge 5810). The pellet was washed (rinsed) with de-ionized water to remove soluble compounds such as salts, soluble COD, from the biomass and then centrifuged again in the same conditions. The obtained pellet was used to measure the TSS and VSS content.

3.2.2.2. pH

The pH of the samples was measured using the pH meter Consort C535.

3.2.2.3. Dissolved oxygen (DO)

The dissolved oxygen of the reactor was measured using the meter WTW Oxi 315i.

3.2.2.4. Total and soluble chemical oxygen demand (COD)

The chemical oxygen demand (COD) is a reference for the content of organic material that can be determined by chemical means.

Total COD (CODt) was determined by means of a conventional method based on the oxidation of organic matter by potassium dichromate ($K_2Cr_2O_7$), a strong standard oxidant, and the colorimetric determination of the residual color by means of titration [151].

In this method, a certain amount of sample (previously diluted 1/10), de-ionized water up to a total volume of 20 mL, 10 mL of $K_2Cr_2O_7$ solution (0.25 N), 0.4 g of $HgSO_4$ and 30 mL of sulphuric acid-silver sulphate solution were introduced into a destruction tube, including the blank, which contained 20 mL of de-ionized water and no sample. Along with a reflux air cooler on top of each destruction tube, all the tubes were placed in a heating apparatus (Tecator 2020 Digestor) at 150°C for 2 hours. After that incubation, the excess of $K_2Cr_2O_7$ was titrated with $Fe(NH_4)_2(SO_4)_2$ solution (0.0625 N) using ferroine (1,10-ortho.fenantroline-monohydrate and Fe-sulphate-7H_2O) as an indicator.

Furthermore, a titer was made in order to determine the normality (N) of the iron ammonium sulphate solution. The titer was composed of ±100 mL of de-ionized water, 10 ml of $K_2Cr_2O_7$ and 20 mL of H_2SO_4. The titer does not need a destruction process; however the indicator must be added before the titration.

Soluble COD (CODs) measurement followed the same method, but the sample was previously centrifuged at 10000 rpm for 10 min (Eppendorf Centrifuge 5810), in which the supernatant was centrifuged again in the same conditions.

3.2.2.5. Kjeldahl nitrogen (TKN)

In determining the Kjeldahl nitrogen (TKN), the Kjeldahl method was followed. This method is based on the conversion of organic nitrogen to ammonium nitrogen under the form of $(NH_4)_2SO_4$, by means of a destruction using concentrated sulphuric acid and copper sulphate as a catalyst at

higher temperatures (400°C). Potassium sulphate is added to raise the boiling point. The ammonium is released in alkaline solution as ammonia, distilled and bound as borate. The content is determined based on an acid-base titration.

Using this method, a Kjeldahl tablet and 10 mL of concentrated H_2SO_4 slowly was added to a certain volume of sample, by means of the dispenser. Then, all the destruction tubes were collected and were taken into the destruction device at 400°C. The suction system (with the suction device) was put on top of the destruction tubes and a digestion step occurred during the following hour. After cooling down, the procedure of the distillation apparatus (Vapodest VAP30 from Gerhardt) was followed by the manual apparatus, i.e. the digested sample was connected to the distillation apparatus in which the ammonia was captured in a boric acid indicator (an acid solution at pH 5.3), as (NH_4BO_3), and titrated with a 0.02 M solution of HCl until a pH value of 5.3 was reached. The titration was performed using a Titronic apparatus from SCHOTT Instruments.

3.2.2.6. Total ammonium nitrogen (TAN)

The total ammonia nitrogen (TAN or NH_4^+-N) measuring was carried out according to the procedure in which a distillation method was used [151].

In this case, a certain volume of sample (not exceed 20 mL) was distilled (Vapodest VAP30 from Gerhardt) under alkaline conditions (1 g of MgO), in which the ammonia is captured in a boric acid indicator (an acid solution at pH 5.3), such as (NH_4BO_3), and titrated with a 0.02 M solution of HCl until a pH value of 5.3 was reached. The titration was performed using a Titronic apparatus from SCHOTT Instruments.

3.2.2.7. Orthophosphate-phosphor (PO_4^{-3}-P)

Orthophosphate-phosphor (PO_4^{-3}-P) was measured photometrically with a HACH Lange DR3900 Photometer using the commercial kit LCK 049. The samples were previously centrifuged at 10000 rpm for 10 min (Eppendorf Centrifuge 5810).

3.2.2.8. Volatile fatty acids (VFA)

To determine the VFA concentration, a diethylether extraction method was used [151]. In this case, 2 mL of sample, 0.5 mL of H_2SO_4 solution, 0.4 g of NaCl, 0.4 mL of internal standard solution (2-methyl hexanoic acid) and 2 mL of diethylether were placed into a centrifuge tube.

The tubes were then mixed for 2 min and centrifuged at 3000 rpm for 3 minutes (Eppendorf Centrifuge 5810). Subsequently, the etheric layer was transferred into a GC vial, in which the short chain fatty acids (SCFA) were extracted. Finally, the extracts (1 µL of extract) were analyzed using a GC-2014 gas chromatograph from Shimadzu, equipped with an Auto injector AOC-20i and an auto sampler AOC-20s, both from Shimadzu as well.

The temperature profile was set from 110 to 165°C, with a temperature increase of 6°C per minute. The temperature of the injector was 220°C, and the temperature of the sampler was 200°C. Nitrogen was used as a carrier gas.

3.2.2.9. VFA removal efficiency

The VFA removal efficiency was given by the quotient between the volatile fatty acids concentration at point t and the VFA concentration fed.

$$VFA\ removal\ efficiency\ at\ point\ t = \frac{VFA_{point\ t}}{VFA_{fed}}\ (\%) \qquad \text{(Eq. 3.1)}$$

3.2.2.10. VSS/TSS ratio

The VSS/TSS ratio was given by the quotient between the volatile suspended solids concentration and the total suspended solids concentration.

$$VSS/TSS = \frac{\mathrm{VSS}}{\mathrm{TSS}}(\%) \qquad \text{(Eq. 3.2)}$$

3.2.2.11. Lipid content

The lipid content was given by the quotient between the lipid concentration and the volatile suspended solids concentration.

$$Lipid\ content = \frac{[\mathrm{Lipid}]}{\mathrm{VSS}}(\%) \qquad \text{(Eq. 3.3)}$$

3.2.2.12. Sludge yield

The sludge yield was given by the quotient between the volatile suspended solids concentration formed and the total amount of COD added.

$$Sludge\ yield = \frac{\text{VSS}}{\text{COD}}(\%) \quad \text{(Eq. 3.4)}$$

3.2.2.13. Lipid production

The lipid production was given by the quotient between the lipid concentration formed and the total amount of COD added.

$$Lipid\ production = \frac{[\text{Lipid}]}{\text{COD}}(\%) \quad \text{(Eq. 3.5)}$$

3.2.2.14. PHA/TSS ratio

The PHA/TSS ratio was given by the quotient between the PHAs concentration and the total suspended solids concentration.

$$PHA/TSS = \frac{\text{PHA}}{\text{TSS}}(\%) \quad \text{(Eq. 3.6)}$$

3.2.2.15. PHA/VSS ratio

The PHA/VSS ratio was given by the quotient between the PHAs concentration and the volatile suspended solids concentration.

$$VSS/TSS = \frac{\text{PHA}}{\text{VSS}}(\%) \quad \text{(Eq. 3.7)}$$

3.2.2.16. Hydraulic retention time

The Hydraulic Retention Time (HRT) was given by the quotient between the volume of the reactor and the flow rate of the influent used.

$$HRT = \frac{Volume}{\text{Flow rate}}(\%) \quad \text{(Eq. 3.8)}$$

3.2.2.17. Sludge retention time

The Sludge Retention Time (HRT) was given by the quotient between the volume of the reactor that contains sludge and the flow rate of the influent used.

$$SRT = \frac{Volume}{\text{Flow rate}}(\%) \quad \text{(Eq. 3.9)}$$

3.2.2.18. Organic loading rate

The Organic Loading Rate (B_v) was given by the product between the organic concentration of the influent and the flow rate of the influent used divided by the volume used in the reactor.

$$Bv = \frac{[Influent].Flow\ rate}{\text{Volume}}(\%) \quad \text{(Eq. 3.10)}$$

3.2.2.19. Sludge loading rate

The Sludge Loading Rate was given by the quotient between organic loading rate and the volatile suspended solids concentration present in the reactor.

$$Bx = \frac{Bv}{\text{VSS}}(\%) \quad \text{(Eq. 3.11)}$$

3.2.2.20. Standard plate counts

Two portions of the final solid product were first dissolved in demineralized water at a concentration of 10 g/100 ml. Several dilutions of this solution were made (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}). The non-diluted solution and the different dilutions were all plated out in Petri dishes, on a selective medium. They were plated out on a Mc Conckey agar (selective medium for *E. coli*) and on Trypticase Soy Agar (TSA, selective medium for total aerobic micro-organisms). All Petri dishes were incubated at 37°C for 24 hours. After 24 hours of incubation, the colony forming units were counted. From the plate counts, the number of *E. coli* and the number of total aerobic organisms in the original solution was calculated, taking into account the dilution factor.

3.2.3. PILOT SCALE SET-UP AND OPERATION

Three different reactors were coupled: the SBR, the Fill-Up Reactor (FUR) and the Decanter. In these tests, an activated sludge from a SBR wastewater treatment of a brewery in Ghent, Belgium, was used as the microbial source. The SBR was fed with the synthetic medium 1 and the FUR was fed with the synthetic medium 2. This reactor was operated for several months, in which different parameters such as solid retention time (SRT), hydraulic retention time (HRT), organic loading rate (Bv) and sludge loading rate (Bx) were varied in time. A process at pilot scale was operated at Avecom NV and the final configuration is represented in Figure 3.1.

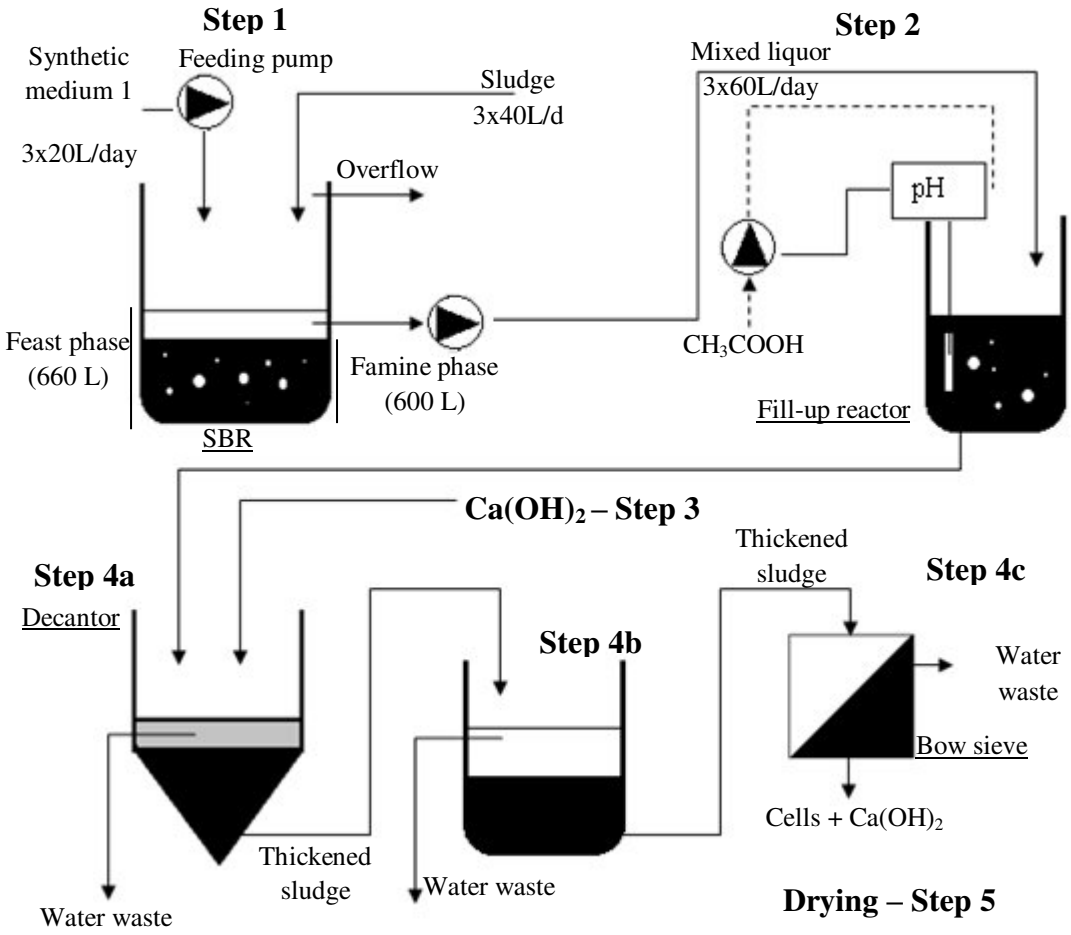

Figure 3.1: General schematic representation of Ca-ROP production.

In this process at pilot scale, five main steps were necessary to obtain the final product. **Step 1** was carried out in a sequencing batch reactor (SBR). The main goal was to select the cells present in the brewery sludge that easily store intracellular carbon resources such as PHA. The SBR system was operated with 3 cycles per day of 7,5 hours in a feast and famine regime. The cycles began with the intake of two different influents (feast phase), one of them had 10 g COD/L (feed) and the other one contained fresh activated sludge from the brewery. After the sludge had consumed all COD the famine phase started. The duration of the feast phase was around 2.5 hours and the duration of the famine phase was around 5 hours. The reactor was continuously aerated. During a feast phase the cells stored intracellular carbon resources, which can then be used as energy source during a famine phase. Three times per day, before the start of the feast phase on SBR system, about 60 L of mixed liquor from the growth reactor was pumped to the FUR.

After 3 cycles of feast/famine regime, the aeration stopped and the mixed liquor was settled (settling phase). After 44 minutes of settling, the supernatant was removed (decanting phase), until the volume of the reactor reached 600 L. Finally, the aeration restarted and resumed the 3 cycles per day of feast/famine regime. The growth reactor was kept at room temperature, around 20°C. The aeration was important to have enough concentration of dissolved oxygen (DO), as well as to have well-mixed liquor. The Table 3.2 gives an overview of the process parameters of **step 1** – SBR system.

Table 3.2: Process parameters of the SBR system in the production of Ca-ROP

Parameter		Unit	Value
Reactor volume	Feast phase	L	660
	Famine phase	L	600
Number of cycles		cycles/d	3
Influent 1 flow (it contains feed)		L/cycle	20
		L/d	60
		L/($L_{reactor}$.d)	0,1
Influent concentration		g COD/L	10
		g VFA-COD/L	10
Organic loading rate (B_v)		g COD/($L_{reactor}$.d)	1
Hydraulic Retention Time (HRT)		d	3.3
Solids Retention Time (SRT)		d	3.3
Influent 2 flow (it contains activated sludge)		L/cycle	40
		L/day	120
		L/($L_{reactor}$.d)	0.2
		g TSS/L	4.5
		g TSS/cycle	180
		g TSS/d	540
		g VSS/L	3
		g VSS/cycle	120
		g VSS/d	360
pH		-	6.5 - 7.5
Dissolved oxygen (feast phase)		mg O_2/L	2.0-4.0
Dissolved oxygen (famine phase)		mg O_2/L	6.0-8.0
Temperature		°C	20

d: day; COD: Chemical Oxygen Demand; VFA: Volatile Fatty Acids; VFA-COD: content of Chemical Oxygen Demand in the Volatile Fatty Acids

Figure 3.2 shows a photograph of the selector reactor set-up that was used in step 1.

Figure 3.2: Photograph of the selector reactor set-up.

Step 2 consisted in enriching the mixed liquor from the growth reactor with PHA using a fill-up reactor (FUR). The sludge from the SBR system was discontinuously harvested and then filled-up in a reactor of 300 L. During the fill-up phase, the biomass was supplied with acetic acid (synthetic medium 2), using a pH adjuster, where nutrients were deficient, to maximize the storage of carbon reserves (lipid/PHA). The mixed liquor was kept under limiting conditions of ammonium and phosphate. The supply of carbon source (influent 2) was pH controlled: in case the pH was above 7.2, the acid influent 2 was dosed until pH 7.0. As the bacteria converted the acetic acid was converted and the pH in the FUR increased again as a result of the bacterial activity. The FUR was kept at room temperature, about 20°C. Three times per day, before the start of the feast phase on SBR system, about 60 L of mixed liquor was pumped from the growth reactor to FUR. This reactor was mixed and aerated intensively to provide sufficient DO. Table 3.3 gives an overview of the process parameters of **step 2** – fill-up phase.

Table 3.3: Process parameters of the fill up phase in the production of Ca-ROP

Parameter	Unit	Value
Reactor volume	L	60-180
Hydraulic Retention Time (HRT)	d	0.33-1
Solids Retention Time (SRT)	d	0.33-1
pH control solution concentration	g COD/L	40
	g VFA-COD/L	40
Fill-up phase duration	h	24

Figure 3.3 shows a photograph of the fill-up reactor set-up with the pH adjuster that was used in step 2 for PHA production.

Figure 3.3: Photograph of the Fill up reactor set-up.

The goal of **step 3** was to stabilize the biomass. About 180 L of mixed liquor per day was pumped to a decanter where Calcium Hydroxide ($Ca(OH)_2$) was added (step 3) at a dosage of 3 g $Ca(OH)_2/L_{mixed\ liquor}$. The mixed liquor was well mixed to ensure that all calcium hydroxide was dissolved.

Figure 3.4 shows a photograph of the decanter that was used in steps 3 and 4.

Figure 3.4: Photograph of the decanter.

The aim of **step 4** was to reduce the volume and thicken the biomass. Different approaches were used to separate the water waste from the end product. Primarily, a decanter was used to remove a first supernatant (**step 4a**). Then, the resultant mixed liquor was placed in large vessels and let to settle for about 1 hour. In addition, the decantation was proceeded by rejection of the supernatant (upper layer) to subsequently obtain a thickened biomass (**step 4b**), which in the end was sieved using a bow sieve with pores of 1 mm (**step 4c**).

Figure 3.5 shows a photograph of the bow sieve that was used in step 4.

Figure 3.5: Photograph of the bow sieve set-up.

The sieved product was dried at 60°C using hot air (**step 5**). Finally, the dry product obtained was pulverized into powder. This powder was stored dry at room temperature.

Figure 3.6: Photograph of the final product, Ca-ROP.

3.2.4. ANALYSIS OF PHA

During the last days of the operational period of the pilot scale tests, a grab sample (ROP – Refined Oil and Protein) was taken upon the filling-up step. Subsequently, experiments were carried out in Avecom NV to develop reproducible and accurate methods to quantify PHA.

3.2.4.1. Methods developed to analyze PHA

3.2.4.1.1. Solvent extraction: Chloroform

This method was used to extract PHAs using chloroform as a solvent to solubilize the PHAs present in the sample. A certain volume of sample was centrifuged using falcon tubes at 10 000 rpm for 15 minutes (Eppendorf Centrifuge 5810). The supernatant was discarded and the pellet rinsed, followed by another centrifugation in the same condition, after which the supernatant was discarded again. The pellet was washed from the falcon tube into schott bottle with 30 mL of chloroform (10 mL/0,1 g of pellet). The samples were then incubated overnight at 37°C, under constant stirring. During next day, the samples were vacuum filtered using fiber glass filters (0.45 µm of pore size, binding agents free) in order to withdraw the non-dissolved biomass, washing the schott bottles and filters with an additional 5 mL of chloroform. The chloroform solution containing the dissolved PHA was then poured into pre-weighed crucibles and the filtration flask was rinsed with methanol.

The chloroform and methanol in the crucibles were left to dry overnight at room temperature in order to evaporate both the chloroform and methanol, and were further dried at 105°C to remove water using the oven Memmert for ca. 24 hours. After cooling down, the crucible was weighted and the PHA content was given by the difference between the porcelain crucible with the dry matter and the porcelain crucible as such divided by the volume of the sample.

3.2.4.1.2. Solvent extraction: propylene carbonate

This method was carried out to extract PHA using propylene carbonate as a solvent to solubilize the PHA present in the sample. A certain amount of the sample was centrifuged using falcon tubes at 10,000 rpm for 15 minutes (Eppendorf Centrifuge 5810) after which the supernatant was discarded and the pellet rinsed with demineralized water. The solution was submitted to another centrifugation in the same condition, after which the supernatant was discarded again.

The pellet was then washed from the falcon tube into a COD tube with 20 mL of propylene carbonate and incubated at 130°C, for at least 15 minutes, with periodic shaking. These solutions were vacuum filtered afterwards with fiber glass filters (0.45 µm of pore size, binding agents free), pre-warmed with propylene carbonate at 130°C. The COD tubes and filters were washed with an additional 20 mL of warm propylene carbonate. The filtered solution was stored in schott bottles and the PHA was precipitated with 4 volumes of cold methanol.

After one day, the precipitated PHA was vacuum filtered with pre-weighted fiber glass filters. The cakes were washed with methanol in order to remove traces of propylene carbonate and dried overnight at 105°C using the oven Memmert for ca. 24 hours. After cooling down, the filters were weighted and the PHA content was given by the difference between the filter with the dry matter and filter as such divided by the volume of sample.

3.3. RESULTS AND DISCUSSION

3.3.1. PRODUCTION OF POLYHYDROXYALKANOATES-RICH BIOMASS

The aim of this experiment was to produce cells enriched in PHAs at a pilot scale, using synthetic media, and to store these cells as - Ca-ROP – Calcium – Refined Oil and Protein. Three different reactors were coupled: the Sequencing Batch Reactor (SBR), the Fill-Up Reactor (FUR) and the Decanter.

- **Period from day 1 until day 17**

During the first sixteen days, all the attention was focused on the selector reactor that started to work as a SBR, using the synthetic medium 1 (10 g COD/L). It took two weeks to study the behavior of the microbial source and the set-up performance. During this period, the pH value and the DO were followed on a daily basis. The VFA, sludge TSS and VSS and lipid content were followed on a regular basis (about 2-3 times a week).

The VFA, TSS and VSS concentrations and lipid content analysis were performed in samples from de mixed liquor, which were taken directly from the SBR. Given that the reactor was continuously mixed through the aeration system, the samples were representative for the complete reactor.

In the beginning of the experiment, the reactor was inoculated with sludge from the brewery and worked in three cycles per day. The feed concentration was 40 g VFA/L (80% of acetic acid and 20% of acetate) and the settling time was set at 44 minutes. In the following days, the removal of VFAs was monitored and it was verified that the removal efficiency was not satisfactory (Table 3.4). Consequently, the feed concentration was decreased to 24 g VFA/L (80% of acetic acid and 20% of acetate) on the 5th day. The pH value and the dissolved oxygen were stable during this first period. The pH value before and after the feeding was around 8.4 and 6.1, respectively. The dissolved oxygen (DO) before feeding was around 3 mg O_2/L, above the recommended value of 2.0 mg O_2/L, but after the feeding the DO decreased to low values, between 0.4-1.0 mg O_2/L, because of the high take-up of oxygen by the bacteria.

On day 4, the sludge volume index after 44 minutes of sedimentation was 950 mL/L (5%). This result is insufficient, because about 60 L is removed each day, i.e. about 10% of the volume. Consequently, during this period, more sludge was removed in the effluent in comparison with the growth obtained. This can explain the decrease of the sludge concentration on the day 4 and 5.

Table 3.4: Pilot-scale SBR reactor set-up and results from the period from day 1 to day 5

Parameter	Unit	Day 1	Day 2	Day 3	Day 4	Day 5
Volume feast phase	L	660	660	660	660	660
Volume famine phase	L	600	600	600	600	600
Influent flow	L/d	60	60	60	60	60
Removed flow	L/d	60	60	60	60	60
Number of cycles	cycles/d	3	3	3	3	3
Medium composition	g acetic acid/g VFA	0.8	0.8	0.8	0.8	0.8
pH feast phase	-	6.1	-	-	7.33	-
pH famine phase	-	8.2	8.1	8.4	-	8.22
DO feast phase	mg O_2/L	0.8	-	1.0	0.4	-
DO famine phase	mg O_2/L	-	3.1	-	-	-
Temperature	°C	20	20	20	20	20
Settling time	min	44	44	44	44	44
Sv 44'	mL/L	-	-	-	950	-
VFA in the feed	g/L	40	40	40	40	24
Organic loading rate	g COD/L.d	4	4	4	4	3
Sludge loading rate	g COD/g VSS.d	0,76	0,71	0,59	0,45	0,61

Table 3.4: Continued

Parameter	Unit	Day 1	Day 2	Day 3	Day 4	Day 5
VFA removal efficiency	%	-	77	53	73	70
TSS	g/L	6,28	6,73	8,04	6,96	5,75
VSS	g/L	5,24	5,65	6,78	5,90	4,92
VSS/TSS	%	83	84	84	85	85
Lipid content	%	20	22	20	-	26

On day 8, an overflow of the mixed liquor by unknown reasons occurred. This explains the decrease of the sludge concentration (TSS and VSS). At the same day, a re-inoculation occurred in the reactor. Therefore, the TSS, VSS and lipid content were not followed in this period since new activated sludge was used and needed to adapt to the new conditions. Besides that, it was observed that all the VFA present in the mixed liquor were consumed. Therefore, the feed concentration was increased to 30 g VFA/L (100% acetic acid) on day 9 (Table 3.5). Once again, the settled volume, after 44 minutes of sedimentation was insufficient for a SBR system that removes 10% of its volume each day.

Between day 9 and day 12, insufficient VFA removal efficiencies were reached (60-80%). Therefore, it was decided to decrease the feed concentration from day 13 on, to 10 g VFA/L (100% acetic acid).

Table 3.5: Pilot-scale SBR reactor set-up and results from the period from day 8 to day 12

Parameter	Unit	Day 8	Day 9	Day 10	Day 11	Day 12
Volume feast phase	L	660	660	660	660	660
Volume famine phase	L	600	600	600	600	600
Influent flow	L/d	60	60	60	60	60
Removed flow	L/d	60	60	60	60	60
Number of cycles	cycles/d	3	3	3	3	3
Medium composition	g acetic acid/g VFA	0.8	1	1	1	1
pH feast phase	-	-	7.8	-	7.34	7.23
pH famine phase	-	8.6	-	8.17	-	-
DO feast phase	mg O_2/L	-	-	0.4	0.7	-
DO famine phase	mg O_2/L	5.4	-	-	-	-
Temperature	°C	20	20	20	20	20

Table 3.5: Continued

Parameter	Unit	Day 8	Day 9	Day 10	Day 11	Day 12
Settling time	min	44	44	44	44	44
Sv 44'	mL/L	950	950	1000	1000	1000
VFA in the feed	g/L	24	30	30	30	30
Organic loading rate	g COD/(L.d)	2.4	3	3	3	3
Sludge loading rate	g COD/(g VSS.d)	1,11	0,94	1,26	0,73	0,68
VFA removal efficiency	%	100	79	-	62	71
TSS	g/L	2,62	-	-	6,59	6,96
VSS	g/L	2,17	-	-	4,10	4,40
VSS/TSS	%	83	-	-	62	63
Lipid content	%	13	-	-	21	22

On day 15 (Table 3.6), it could be observed that the VFA present in the mixed liquor were all consumed. The organic loading rate and the sludge loading rate were too low between day 15 and 17. This can be the reason for the decreasing, of the lipid content, in the biomass decreased. In Table 3.6, it can also be seen that the problems with the settling of the sludge had been continuing. After day 17, the set-up for the production of Ca-ROP (Calcium rich – Refined Oil and Protein) was modified.

Table 3.6: Pilot-scale SBR reactor set-up and results from the period from day 15 to day 17

Parameter	Unit	Day 15	Day 16	Day 17
Volume feast phase	L	660	660	660
Volume famine phase	L	600	600	600
Influent flow	L/d	60	60	60
Removed flow	L/d	60	60	60
Number of cycles	cycles/d	3	3	3
Medium composition	g acetic acid/g VFA	1	1	1
pH feast phase	-	7.5	-	-
pH famine phase	-	8.3	8.65	8.64
DO feast phase	mg O_2/L	1.2	1.7	0.6
DO famine phase	mg O_2/L	-	-	-
Temperature	°C	20	20	20
Settling time	min	52	52	52
VFA in the feed	g/L	10	10	10
Organic loading rate	g COD/(L.d)	1	1	1

Table 3.6: Continued

Parameter	Unit	Day 15	Day 16	Day 17
Sludge loading rate	g COD/(g VSS.d)	0.21	-	0.24
VFA removal efficiency	%	100	100	100
TSS	g/L	7,43	-	6,42
VSS	g/L	4,76	-	4,11
VSS/TSS	%	64	-	64
Lipid content	%	13	-	11

On day 11, 15 and 17, fill-up tests using mixed liquor from the SBR at the end of the famine phase were carried out. In these fill-up tests, the pH was controlled at 7.2 using a solution of synthetic medium 2 with 40 g VFA/L. Three tests were performed in order to have a good background of the process for 24 hours. The results of these tests can be observed in Table 3.7.

Table 3.7: Results for sludge concentration and lipid content in the fill-up tests

Day	Unit	Day 11		Day 15		Day 17	
Volume	L	60		60		60	
DO	mg O_2/L	2-4		2-4		2-4	
Temperature	°C	20		20		20	
Influent concentration	g VFA/L	40		40		40	
Volume added of the influent	L	17		14		23	
Time	h	0	24	0	24	0	24
TSS (g/L)	g/L	6.59	6.18	7.43	7.35	6.42	6.77
VSS (g/L)	g/L	4.10	4.76	4.76	5.83	4.11	4.67
VSS/TSS (%)	%	62	77	64	79	64	69
Lipid content	%	21	20	13	34	11	28
Sludge yield	g VSS/g COD $_{added}$	0,18		0,26		0,15	
Lipid production	g lipid/g COD $_{added}$	0,03		0,20		0,09	

As can be seen in Table 3.7, the initial volume was 60 L. The total volume of the feed added after 24 hours was between 14 and 23 L with a concentration of 40 g/L.

Since the pH was controlled at 7.2, the pH during the test was fluctuating around that value. The DO in all tests was kept in a good range, 2-4 mg O_2/L. As can be observed in Table 3.7, the VSS content and the lipid content in the mixed liquor increased after 24 hours. As there was no nitrogen and phosphate added in the fill-up reactor, this increase in VSS is the result of the storage of carbon resources, because part of the VSS is carbon resources (lipid) content.

Concerning the lipid production, yields of about 0.03-0.20 g lipid/g COD added were achieved (Table 3.7). Only the volume of influent added was monitored and the yields were calculated based on the COD added instead of the COD consumed by the activated sludge. This means that possibly the yields obtained were being underestimated. Furthermore, the tests performed on day 15 and 17 showed a great improvement of the lipid content in the sludge after 24 hours filling-up, i.e. the lipid content at the start was 11-13% and in the end of the test 28-34%.

- **Period from day 17 until day 32**

With the knowledge acquired during the previous 17 days, a complete set-up, to produce enriched sludge in PHA at pilot scale, was installed. As described in paragraph 3.3, Ca-ROP production was done in five main steps. Hereafter some of the results obtained with this process will be described and discussed.

  - **Step 1**

The aim of this step was to select the cells present in the activated sludge that easily store intracellular carbon resources as lipid/PHA. To perform that goal a SBR system was used, submitting the activated sludge to consecutive periods of external substrate accessibility ("feast") and unavailability ("famine"), which generates a so-called unbalanced growth. The SBR system was operated with 3 cycles per day of 7.5 hours in a feast and famine regime. A cycle started with the intake of two influents (feast phase), one of them had 10 g COD/L (feed) and the other one contained fresh activated sludge. The famine phase started 2.5 hours after the start of the cycle.

In terms of volumes, per cycle 20 L of influent that contained feed and 40 L of influent that contained fresh activated sludge were added. Before starting a new cycle, 60 L of mixed liquor was pumped from the SBR to the fill-up reactor. So, the volume of the SBR was kept between 660 L (feast phase) and 600 L (at the end of the famine phase).

In Table 3.8, an overview of the results of the analysis made during this period is given for the SBR (the process parameters were shown in Table 3.2).

Table 3.8: Results for the sludge concentration and lipid content in the selector reactor (SBR) at the end of the feast phase (600 L of volume)

Parameter	Unit	Day 22	Day 24	Day 25	Day 29
Volume feast phase	L	660	660	660	660
Volume famine phase	L	600	600	600	600
Influent flow	L/d	60	60	60	60
Removed flow	L/d	60	60	60	60
Number of cycles	cycles/d	3	3	3	3
Medium composition	g acetic acid/g VFA	1	1	1	1
pH feast phase	-	7.8	-	8.0	-
pH famine phase	-	8.3	8.3	8.5	8.2
DO feast phase	mg O_2/L	4.7	6.9	6.9	-
DO famine phase	mg O_2/L	-	-	-	-
Temperature	°C	20	20	20	20
VFA in the feed	g/L	12	12	12	12
Organic loading rate	g COD/(L.d)	1.2	1.2	1.2	1.2
Sludge loading rate	g COD/(g VSS.d)	0.35	0.36	0.40	0.40
VFA removal efficiency	%	100	100	100	100
TSS	g/L	5.74	6.22	5.95	5.15
VSS	g/L	3.46	3.33	2.99	2.98
VSS/TSS	%	60	54	50	58
Lipid content	%	16	24	36	20

As shown in Table 3.8, the pH value and the dissolved oxygen were stable during this period. The pH value in the famine phase was between 8.2 and 8.5, and was between 7.8 and 8.0 after feeding. The dissolved oxygen in the feast phase was between 4.7 and 6.9 mg O_2/L, so the oxygen was not a limiting factor. The temperature was kept at room level, which was about 20°C.

As can be seen in Table 3.8, all acetic acid, at the end of the feast phase, was consumed. During this period, the organic loading rate was 1.2 g COD/(L.d). Moreover, the sludge loading rate kept quite stable, between 0.35 and 0.40 g COD/(g VSS.d). The ratio of VSS on TSS during this process was between 50-60%, which was still a satisfying range since it is an activated sludge. The lipid content in the cells was stable in the range of 16 and 24%.

The sludge concentration in the selector reactor between day 22 and day 29 showed a slight decrease in TSS and VSS. Nevertheless, the sludge concentration during the selection phase was of less importance, as this phase focusses on the selection of the desired organisms.

- **Step 2**

Step 2 aimed to enrich the sludge with lipids or PHA. Per day, about 60 L of the mixed liquor from the selector reactor was pumped to the fill-up reactor, before the start of the feast phase. This way, in every cycle a new batch of sludge was transferred into the fill up reactor. Therefore, the newest sludge remained less time in the reactor than the older one, i.e. the HRT/SRT was between 8 and 24 hours. The influent used in the fill-up reactor had only carbon source. This way, the nitrogen and phosphate was limited in the medium, meaning that these two substrates were not added. The aim of this limited medium was to create a shortage of nutrients and potentiate the lipid/PHA storage in the cells.

Table 3.9: Results for sludge concentration and lipid content in the fill up reactor (FUR)

Parameter	Unit	Day 22	Day 24	Day 29	Day 31
Mixed liquor volume	L	70	180	190	175
Influent concentration	g VFA/L	40	40	40	40
Organic loading rate	g COD/(L.d)	4.6	4.4	7.6	9.1
Sludge loading rate	g COD/(g VSS.d)	1.0	1.0	1.6	2.0
DO	mg O_2/L	0.8	-	1.6	-
TSS	g/L	6.61	6.76	6.55	5,92
VSS	g/L	4.56	4.57	4.76	4,63
VSS/TSS	%	69%	68%	73%	78%
Lipid content	%	37%	37%	38%	63%
Sludge yield	g VSS/g COD_{added}	0.35	0.39	0.35	-
Lipid yield	g Lipid/g COD_{added}	0.30	0.24	0.21	-

As can be seen in Table 3.9, the initial volume of mixed liquor was between 160-190 L, except on the day 22, when it was just 70 L. During this period, the organic loading rate applied was between 4.4 and 7.6 g COD/(L.d) and the corresponding the sludge loading rate was between 1.0 and 2.0 g COD/(g VSS.d).

Since the pH was controlled at 7.2, the pH during this process was fluctuating around that value. In relation to the dissolved oxygen in the reactor, the values obtained were rather low, between 0.8 and 1.6 mg O_2/L, but still high enough to guaranty sufficient oxygen supply. Oxygenation was not a limiting factor.

Comparing the VSS concentration values observed in Table 3.8 and Table 3.9, it can be concluded that an increasing of VSS concentration, during the 24 hours of the filling-up, in the

mixed liquor. As there was no nitrogen and phosphate added in this phase, this growth was a result of the storage of intracellular reserves as lipid/PHA. As the lipid content is part of the VSS, the conclusion can be made that the sludge was enriched with intracellular reserves, even without considering the analysis to determine the lipid content. Before the fill-up phase, the lipid content in the mixed liquor was between 16 and 24% and after 24 hours of the filling-up the lipid content obtained was between 37 and 38%, except on day 31, when the lipid content was 63%. This value is probably an analysis error, because before such high lipid contents were never obtained.

Concerning the sludge and the lipid production, as shown in Table 3.9, good yields in growth of the biomass (sludge yield) and in intracellular reserves storage (lipid yield) were achieved. These yields were calculated based on the COD added, but they could be higher if not all the COD had been removed. The lipid yields obtained were between 0.21 and 0.30 g lipid/g COD. The sludge yields obtained were between 0.35 and 0.39 g VSS/g COD. So, the conclusion can be made that all VSS growth almost exclusively occurred, due to lipid increase.

From previous evaluation of this period, it is possible to conclude that a pH control at 7.2 and good dissolved oxygen concentration in the reactor led to good lipid content in the cells using a feed with just a carbon source, no nitrogen nor phosphate.

- **Step 3**

This step was implemented to stop the biological activity in order to keep the lipid/PHA in the constitution of the cells. To reach this goal, implementing a pH shift can be an effective strategy. Afterwards, to separate the product from the biomass, it would be better if the settling characteristics of the mixed liquor increased. Therefore, a clarifying agent capable to increase the pH until 11-12 was used, to flocculate the cells increasing the settling characteristics. The clarifying agent used was calcium hydroxide ($Ca(OH)_2$) at a dosage of 3 g $Ca(OH)_2/L_{mixed\ liquor}$. At this dosage, the pH increased until around 12, reaching the goal of this step which was to stabilize the biomass.

- **Step 4**

During this step, the biomass was separated from the aqueous phase, obtaining a thickened sludge, suitable to dry quickly. As said before, by increasing the pH of the mixed liquor towards

12 with $Ca(OH)_2$ the settling characteristics of the cells improved. Firstly, after the addition of $Ca(OH)_2$ and the mixing of the mixed liquor in the decanter, about one third of the volume was removed (supernatant) after 15-30 minutes. Then, the resulting liquor was placed in large vessels and settled for about 1 hour. In addition, the resulting supernatant was discarded by decantation. After two consecutive decantations, a filtration of the resultant biomass was performed using a bow sieve.

The dry matter content of the mixed liquor after these two consecutive decantations was around 25 g/L. After the filtration, using a bow sieve of 1 mm pore size, the content of dry matter of the mixed liquor was around 100 g/L.

- **Step 5**

This step consisted in drying and storing the product. The product resulting from the bow sieve was dried using hot air at 60°C. Finally, the dry product was pulverized, obtaining a powder and stored at room temperature, around 20°C. The name given to the final product was Ca-ROP (Calcium rich – Refined Oil and Protein).

3.3.2. CHARACTERIZATION OF THE CA-ROP PRODUCT

During the last days of the production of Ca-ROP at a pilot scale, a grab sample was taken upon the filling-up step. This sample of the filled-up biomass (ROP) was characterized in terms of TSS, VSS, COD and TKN.

At this moment of the R&D, it was mandatory to evaluate if the final product (Ca-ROP) and the filled-up biomass (ROP) contained some polyhydroxyalkanoates (PHA). So, an attempt was made to develop methods reproducible and accurate for quantifying PHA. These methods were inexistent in Avecom NV. Methods were developed during this thesis, making use of the lab infrastructure that was available. The samples Ca-ROP, ROP and sludge as such were analyzed.

3.3.2.1. PHA analysis

Chloroform extraction is currently used [109, 115, 116] as a standard method for PHA recovery at lab scale. This way, this method should be considered reproducible and accurate to quantify PHAs. In Table 3.10 and 3.11 shows the results obtained for chloroform extraction.

Propylene carbonate extraction is also a method found in the literature [111, 115] to recover PHA from cells. Thus, this method was performed using the samples Ca-ROP, ROP and sludge as such. The results obtained are shown in the Table 3.10 and 3.11.

Table 3.10: Amount of PHAs obtained with chloroform and propylene carbonate extraction

Sample	Sample volume (mL)	Amount of Polyhydroxyalkanoates (PHAs) (g/L)	
		Chloroform extraction	Propylene Carbonate extraction
ROP	20	4.71 ± 0.27	6.50 ± 0.34
	10	5.82 ± 1.66	6.66 ± 0,49
	Average	5.27 ± 1,17	6.58 ± 0,36
Sludge as such	20	0.53 ± 0.10	0.08 ± 0.01
	10	0.53 ± 0.16	0.14*
	Average	0.53 ± 0.11	0.10 ± 0.04

*Only one sample taken into account

Table 3.11: Some important ratios determined by chloroform and propylene carbonate extraction

Sample	Ratio	Unit	Chloroform	Propylene Carbonate
ROP	PHA/TSS	%	28	35
	PHA/VSS	%	36	45
Sludge as such	PHA/TSS	%	2,2	0,4
	PHA/VSS	%	5,2	1,0

As can be seen in Table 3.10, the filled-up biomass (ROP) contained PHAs, between 4.7 and 6.7 g/L, as shown by the chloroform and the propylene carbonate extraction. On the other hand, this analysis confirmed that the sludge as such contained a small amount of PHAs in its constitution. In addition, it is possible to verify that the propylene carbonate extraction allowed to obtain a higher amount of PHA from the ROP sample than the ones with chloroform. Furthermore, the methods didn't decrease their sensibility and reproducibility when different volumes of sample were used.

In Table 3.11, it can be observed that the obtained biomass had a high content of PHA in its constitution. By the chloroform extraction, the PHA content at the start (sludge as such) was about 5.2%, while the PHA content after filling-up was about 36%. By the propylene carbonate extraction, the PHA content at the start (sludge as such) was about 1.0% while the PHA content after filled-up was about 45%. These results confirmed that the sludge was enriched in lipids/PHAs as was pretended, reaching the goal successfully.

Following the chloroform and propylene carbonate extraction, it was not possible to characterize the final product Ca-ROP, because non reliable results were obtained. Besides that, it is expected that the PHA was kept in the cells. Probably, the calcium presence had interfered with the analysis performed.

3.3.3. THEORETICAL COMPOSITION OF THE FINAL CA-ROP PRODUCT

The composition of the Ca-ROP was calculated based on the characteristics of the biomass harvested from the fill-up phase and taking into account the addition of $Ca(OH)_2$ in the decanter at a dosage of 3.0 g $Ca(OH)_2/L_{mixed\ liquor}$. In case all calcium was precipitated as $CaCO_3$ during drying, one can calculate that 4.05 g $CaCO_3$ could be produced per liter of sludge because the molecular weight of $CaCO_3$ is 1.35 times higher than the molecular weight of $Ca(OH)_2$. Table 3.12 summarizes the theoretical composition and the results obtained for the main characteristics about Ca-ROP.

Table 3.12: Characterization of the Ca-ROP

Parameter	Unit	Ca-ROP[5]	Theoretical composition
Presence[1] of			
- E. Coli	CFU/mL	Not detected	-
- Aerobic microorganisms	CFU/mL	Not detected	-
Dry solids	% (g/g Ca-ROP)	90.7	-
Volatile solids	% (g/g Ca-ROP)	30.7	-
	% (g/g DS)	33.9	45
Ash	% (g/g Ca-ROP)	60.0	-
	% (g/g DS)	66.1	55
Calcium	% (g/g Ca-ROP)	19.9	-
	% (g/g DS)	21.9	15
as CaCO3	% (g/g DS)	54.8	-
PHA[2]	% (g/g Ca-ROP)	Not detected	17.4
	% (g/g DS)		19.2
PHA[3]	% (g/g Ca-ROP)	Not detected	21.8
	% (g/g DS)		24.0
TKN	mg/g Ca-ROP	24.0	-
	mg/g DS	26.4	26
Protein content[4]	% (g/g Ca-ROP)	15	-
	% (g/g DS)	17	16

[1] – Microbial analysis was performed using standard plate counts; [2] – Content of PHA analyzed by chloroform extraction; [3] – Content of PHA analyzed by propylene carbonate extraction; [4] – Protein content assuming a conversion factor of 6.25 g protein/g TKN; [5] – Average values of 2 samples

As can be seen in Table 3.12, the presence of *E. coli* or another aerobic microorganism was not detected in the final product. This means that there are probably no other bacteria growing after the Ca-treatment. Moreover, Ca-ROP is a very dry product, with a dry matter content of about 90%. The product has high ash content (about 66% on dry matter), more than was expected, around 55%. About one third of the ash is calcium. Supposing that all calcium is present as calcium carbonate, this would mean that about 55% of the dry matter in the product is $CaCO^3$.

The protein content of the calcium rich product is about 15%, as was predicted. As said before, non-reliable results were obtained for the PHA presence in the final product – Ca-ROP. Assuming that the PHA content in the cells was kept at the same level after the addition of the calcium $Ca(OH)_2$, the conclusion can be made that the PHA content of the final product exists between 17 and 22%. Moreover, it appears that the results of the analysis for PHA corresponds to the other results, i.e. the % ash content plus the % protein content plus the % PHA content is less than 100%. Therefore, these results proved and validated the methods developed to analyze the PHA, showing that they are reproducible and reliable methods.

3.4. CONCLUSIONS

First of all, the major goal was accomplished with success. About 25 kg of Ca-ROP was produced to be used in trials in view of a possible application. Ca-ROP is the final product obtained, namely biomass enriched in PHAs.

A complete set-up was installed to produce enriched sludge in PHA at pilot scale with success. This set-up contemplated all the general steps needed to be implemented for PHA production at industrial scale as mentioned in Figure 2.7. Those steps are fermentation, cell precipitation, press filtration, drying and powdering. The fermentation step consisted in the selection of the desirable organisms from the activated sludge used, where a feast and famine regime was applied, and in the enrichment of the biomass with PHA, where an inexistence of nutrients and an excess of carbon source (acetic acid) was used. The TSS concentration during fermentation was between 5.15 and 6.76 g/L while the VSS concentration was between 2.98

and 4.76 g/L. During the production the lipid content were monitored using an analysis already used before in the company for the same purpose obtaining fast results as was demanded. By this analysis, the lipid content in the end of the fermentation was 37-38%. During the enrichment of the biomass with PHA the sludge yields and the lipid yields were calculated. The lipid yields and the sludge yields were obtained between 0.21 and 0.30 g lipid/g COD and 0.35 and 0.39 g VSS/g COD, respectively. So, it can be concluded that all VSS growth practically occurred, due to lipid increase. Cell precipitation step was conducted in a decanter where was added calcium hydroxide was added to stabilize the cells and to improve their settling characteristics. Press filtration step was executed using a bow sieve with 1 mm of pore size, thickening 5 times the mixed liquor. The drying step was performed using hot air at 60°C. Finally, the powdering step was fulfilled pulverizing the dry product and stored at room temperature.

After Ca-ROP production the final product was characterized to evaluate the presence of PHA. Two methods were developed during this thesis that were inexistent in Avecom NV to analyze PHA. In the end, the results obtained through these methods were consistent with the ones obtained during the Ca-ROP production that were called lipid content. Through the chloroform extraction the PHA content of the biomass in the end of fermentation step was around 36% while with the propylene carbonate extraction was around 45%. Those methods were used to characterize the final product Ca-ROP, but non reliable results were obtained.

The final product is a very dry product, with a dry matter of about 90%. The protein content of the calcium rich product is about 16%. The PHA content of the final product is between 17 and 22%.

CHAPTER 4

PRODUCTION OF RESERVE COMPOUNDS FROM VEGETABLE PROCESSING WASTE STREAMS BY (OPEN) MIXED CULTURE

4.1. SUMMARY AND MOTIVATION

The majors factors limiting the development of a process in view of an industrial implementation are the composition and the costs of the substrates used, which will influence the production cost of the resulting product. Therefore, using complex media that can have a low cost or even a negative cost are the best options to have an economically feasible process. Thus, some experiments were performed at lab scale to produce a sludge enriched in PHA or another type of storage compound like triacylglycerols or wax esters. The complex media used were obtained from a Dutch vegetable processing company, dealing with a quantity of vegetable waste excess streams of the order of 50 ton COD per day. They might be suited as an influent for the production of reserve compounds, which, on their turn, can be used for tests in view of a possible application.

First of all, the complex media was subjected to a chemical characterization. Afterwards, batch tests were performed to evaluate if the full COD content was consumed or only the volatile fatty acids were converted. Then, some tests were done to shorten the production time of reserve compounds or to increase the accumulation of these compounds, and subsequently, to enhance the productivity, optimizing the process of production. This work was developed at Avecom NV as well.

4.2. MATERIAL AND METHODS

4.2.1. INOCULUM AND SUBSTRATES

4.2.1.1. Inoculum

Activated sludge from a SBR wastewater treatment of a brewery in Ghent, Belgium, was used as the microbial source to procedure the Quick Scans.

4.2.1.2. Substrates

In these experiments a complex medium was used as substrate. The complex medium used came originally from a Dutch company, however different samples were used, and therefore, the compositions had slight differences due to the fact that samples were taken at different time periods. These samples were vegetable streams that were acidified in acidification tanks and sieved. Several sieves were used: 1 mm, 0.5 mm and 0.1 mm. The samples were labeled as D, F, L and N. The samples N and L were taken on October 22, the sample D was taken on October 17 and the sample F was taken on October 18. The samples D and N were obtained by a sieve with a size of 0.5 mm, while the samples L and F were obtained by a sieve with a size of 1.0 mm.

Upon reception of those complex media a chemical characterization was done, evaluating some parameters, such as: total chemical oxygen demand (COD_T), soluble chemical oxygen demand (COD_S), total suspended solids (TSS), volatile suspended solids (VSS), dry matter (DM), volatile solids (VS), ash, total ammonia nitrogen (TAN), Kjeldahl nitrogen (TKN), orthophosphate-phosphor (PO_4^{-3}-P) and volatile fatty acids (VFA). The VFA analyzed were acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid and caproic acid. Remark: The analytical techniques were already described in section 3.2.2.

By lack of a good, reliable and fast method to analyze bacterial lipid content, it was chosen to follow the VSS content of the sludge during the tests. After all, the lipid content is part of the VSS. If a good VSS yield would not be achieved, subsequently the conclusion could be made that neither a good lipid yield was obtained.

4.2.2. BATCH EXPERIMENTS

A series of 5 experimental runs, denominated "quick scans", were performed in lab-scale batch reactors. The first quick scans aimed to study if the COD content in the complex media was uptake, optimizing the process. In the last quick scans (quick scan 4 and 5) the nutrient uptake was monitored and the balance of COD/N/P uptake was calculated to estimate the possible lipid accumulation.

4.2.2.1. Quick scan 1

In order to evaluate influent D, a quick scan was done, using activated sludge from a SBR treating brewery wastewater. This quick scan consisted of 2 steps. The first step was an adaptation phase. This phase was introduced to select the organisms that easily store intracellular carbon sources such as lipids or PHA. The second step was a fill-up phase. In this phase, it was attempted to enrich the selected organisms in lipids or PHA.

4.2.2.1.1. Adaptation phase

The adaptation phase was done in lab-scale batch reactors (R_1 and R_2) with an active volume of 1L.

The reactors R_1 and R_2 were placed on a shaker (120 rpm) at room temperature. They were continuously aerated in order to have a high concentration of dissolved oxygen (DO). The pH was manually adjusted between 6.5 and 7.5 by the addition of a solution of hydrochloric acid (HCl) 1 N. For three days, the sludge was subjected to a feast and famine regime, in cycles of 8 h, or 3 cycles per day.

As influent D was only partially acidified (VFA/CODt ~13%), two tests were done with influent D. Reactors R_1 and R_2 were both supplied with sample D, but with a different influent flow rate. Reactor R_1 was loaded with influent D, assuming that only the VFA would be converted and not taking the other COD into account. Reactor R_2 was loaded with influent D, taking the full COD content of the influent into account. The process parameters for the reactors in the adaptation phase are summarized in Table 4.1.

Table 4.1: Overview of the process parameters of the 2 batch reactors in the adaptation phase

Parameter	Unit	R_1	R_2
Reactor volume	L	1	1
Cycles			
- frequency	Cycles/day	3	3
- total duration	h	8	8
- feast	h	2.5	2.5
- famine	h	5.5	5.5
Influent		Influent D	Influent D
- COD	g/L	54,5	54,5
- VFA	g/L	8	8.0
Influent flow rate	L/L.d	0.18	0.30
Sludge concentration	g TSS/L	20.0	20.0
	g VSS/L	7.74	7.74
Volumetric loading rate	g COD/(L.d)	10.0	**1.65**
	g VFA-COD/(L.d)	**1,45**	0.24
Sludge loading rate	g COD/(g VSS.d)	1.29	**0.21**
	g VFA-COD/(g VSS.d)	**0.19**	0.03
pH			
upon feeding	-	7.2	7.4
- in feast	-	7.8	8.0
- in famine	-	8.15	8.4
DO			
- in feast	mg O_2/L	1.0-2.0	6.0-7.0
- in famine	mg O_2/L	1.5-3.0	6.0-8.0

During the adaptation phase, the evolution of VFA and soluble COD in the reactors was followed during 1 cycle, on the 2nd day of adaptation. This was done to evaluate the presence of a famine phase, as the presence of a famine phase is deemed necessary for the selection of organisms that are able to accumulate reserve lipids.

4.2.2.1.2. Fill-up phase

In this phase, the main goal was to enrich the sludge using complex media with lipids or PHAs.

The mixed liquor from the reactors R_1 and R_2 was filled-up in a reactor with 400 mL of active volume, after 3 days of adaptation phase. The pH was controlled by PC-Panel μDCU software and a pH meter Easyferm plus K8 425, Hamilton Bonaduz AG. The pH in both reactors was controlled by a main system from Biostat® A plus, by Sartorius BBI Systems. The pH was controlled below 7.2 by addition of fresh influent D. This set-up was kept at room temperature, around 20°C. The reactors used in the fill-up tests were continuously aerated to have a good

concentration of dissolved oxygen (DO) and also to have a well-mixed medium. The fill-up tests were performed for 24 hours. The consumption of influent D was also monitored.

4.2.2.2. Quick scan 2

In this experiment a quick scan was done using the same microbial source (activated sludge from a SBR treating brewery wastewater) but with another medium, influent N, with a slight different composition compared to the influent D that was used as substrate in Quick scan 1.

Furthermore, the duration of the adaptation phase was evaluated to obtain a better enrichment of lipids or PHAs in the sludge.

This quick scan consisted of 2 steps: an adaptation phase for selection (varying between 0 and 3 days) and a fill-up phase using mixed liquor with 1 and 3 days of adaptation phase.

4.2.2.2.1 Adaptation phase

The adaptation phase was done in lab-scale batch reactors (R_1 and R_2) with an active volume of 1L. The set-up installed was similar to the one described in section 4.2.2.1.1.

As said previously, the influents (vegetable streams) were only partially acidified (VFA/CODt ~13%). So, two tests were done with the influent N: reactors R_1 and R_2 were both supplied with the sample N, but with a different influent flow rate. Reactor R_1 was loaded with influent N, assuming that only the VFA would be converted and not taking the other COD into account. Reactor R_2 was loaded with influent N, taking the full COD content of the influent into account.

The process parameters for the reactors in the adaptation phase are summarized in Table 4.2.

Table 4.2: Overview of the process parameters of the 2 batch reactors in the adaptation phase

Parameter	Unit	R_1	R_2
Reactor volume	L	1	1
Cycles			
- frequency	Cycles/day	3	3
- total duration	h	8	8
- feast	h	2.5	2.5
- famine	h	5.5	5.5
Influent		Influent N	Influent N
- COD	g/L	74.29	74.29
- VFA	g/L	9.72	9.72

Table 4.2: Continued

Parameter	Unit	R_1	R_2
Influent flow rate	L/(L.d)	0.16	0.025
Volumetric loading rate	g COD/(L.d) g VFA-COD/(L.d)	11.89 **1,56**	**1.86** 0.24
Sludge loading rate	g COD/(g VSS.d) g VFA-COD/(g VSS.d)	0.96 **0.13**	**0.15** 0.03
pH **upon feeding** **- in feast** **- in famine**	 - - -	 6.5-8.0 6.5-8.0 6.5-8.0	 6.5-8.0 6.5-8.0 6.5-8.0
DO **- in feast** **- in famine**	 mg O_2/L mg O_2/L	 2.0-4.0 3.0-4.0	 4.0-6.0 6.0-8.0

During the adaptation phase, the evolution of VFA and soluble COD in the reactors was followed, during 1 cycle, on the 2nd day of adaptation. This was done to evaluate the presence of a famine phase, as the presence of a famine phase is deemed necessary for the selection of lipid accumulating organisms.

4.2.2.2.2. Fill-up phase

In these experiments, the goal was to investigate the minimal duration of the adaptation phase necessary to allow a good fill-up of the sludge using complex media.

The test was done using the same set-up as described in section 4.2.2.1.2. The mixed liquor from the reactors R_1 and R_2 was filled-up in a reactor with 250 mL of active volume after 1 and 3 days of adaptation phase. The pH was controlled below 7.2 by addition of fresh influent N. The fill-up tests were performed for 24 hours. The consumption of the influent N was also monitored.

4.2.2.3. Quick scan 3

The aim of this experiment was to evaluate whether the activated sludge from the SBR could fill-up with a vegetable processing stream, without an adaptation phase. Therefore, this third quick scan consisted only of 1 step: a 24-hour fill up phase.

4.2.2.3.1. Fill-up phase

An activated sludge from a SBR treating brewery wastewater was directly filled-up in four reactors with 400 mL of volume. There was no adaptation phase. Each reactor was supplied with four different vegetable streams: influent D, influent N, influent L and the influent F. The test was done using the same set-up as described in paragraph 4.2.2.1.2. The pH was controlled below

7.2 by addition of fresh influent. The fill-up tests were performed for 24 hours. The consumption of the influents was also monitored.

4.2.2.4. Quick scan 4

The aim of this experiment was to evaluate the influence of the pH set point used in the fill-up tests. This quick scan consisted of a single step – a 24 hour fill up phase at a different pH set point: 8.2.

4.2.2.4.1. Fill-up phase

An activated sludge from a SBR treating a brewery wastewater was directly filled-up, without an adaptation phase, in two reactors with 400 mL of volume. Each reactor was supplied with a different vegetable stream: influent L and influent F. The test was done using the same set-up as described in section 4.2.2.1.2. The pH was controlled below 8.2 by addition of fresh influent. The fill-up tests were performed for 24 hours. The consumption of the influents was also monitored.

4.2.2.5. Quick scan 5

The aim of this experiment was to evaluate the effect of a shorter fill-up phase to produce neutral lipids. This quick scan consisted of a single step: an 8-hour fill up phase.

4.2.2.5.1. Fill-up phase

An activated sludge from a SBR treating brewery wastewater was directly filled-up in two reactors with 400 mL of active volume, without an adaptation phase. Each reactor was supplied with a different vegetable stream: influent N and influent F. The test was done using the same set-up as described in section 4.2.2.1.2. The pH was controlled below 7.2 by addition of fresh influent. The fill-up tests were performed for 8 hours. The consumption of the influents was also monitored.

4.3. RESULTS AND DISCUSSION

4.3.1. COMPLEX MEDIA CHARACTERIZATION

Upon reception of the samples delivered by the Dutch company, a chemical characterization was done. The samples were vegetable processing streams that were acidified in the acidification tanks and sieved. Sieving was deemed necessary to remove suspended solids from the acidified streams, as they might interfere in the lipid production. Several sieves were used: 1mm, 0.5mm

and 0.1 mm. Table 4.3 summarizes the characteristics of the samples D, N, L and F and in Table 4.4 are presented some important ratios calculated with these parameters.

Table 4.3: Characteristics of the influent D, N, L and F

Parameter	Unit	Influent D 17/10 – 0.5 mm	Influent N 22/10 – 0.5 mm	Influent L 22/10 – 1.0 mm	Influent F 18/10 – 1.0 mm
pH	-	3.65	3.45	3.45	3.49
CODt	mg /L	54489	74289	79591	120407
CODs	mg/L	47347	69101	71836	95101
CODt-CODs	mg/L	7142	5188	7755	25306
TSS	g/L	3.86	5.27	8.04	14.2
VSS	g/L	3.38	4.67	7.16	11.9
DM	g/L	34.3	47.3	46.0	70.0
VS	g/L	26.4	40.3	39.1	62.5
Ash	g/L	7.84	7.01	6.89	7.49
TKN in solution	mg/L	339	622	622	630
NH_4^+- N	mg/L	132	162	178	203
P in solution	mg/L	149	164	164	120
VFA					
Acetic acid	mg/L	6200	8056	8740	6558
Propionic acid	mg/L	4339	6379	7187	4179
Isobutyric acid	mg/L	656	535	93	9750
Butyric acid	mg/L	0	0	0	0
Isovaleric acid	mg/L	456	670	1022	952
	mg/L	0	27	0	0
Valeric acid	mg/L	387	323	61	453
	mg/L	0	0	0	0
Isocaproic acid	mg/L	362	122	377	399
Caproic acid					
VFA-COD	mg/L	7981	9719	10523	8687

CODt – Total chemical oxygen demand; CODs – Chemical oxygen demand of the soluble compounds; TSS – Total suspended solids; VSS – Volatile suspended solids; DM – Dry matter; VS - Volatile solids; TKN – Kjeldahl nitrogen; P – Phosphorous; VFA – Volatile fatty acids; VFA-COD – Chemical oxygen demand content of the volatile fatty acids.

As can be seen in Table 4.4, samples N and L were taken on October 22, sample D was taken on October 17 and sample F was taken on October 18. The samples D and N were obtained by a sieve with a size of 0.5 mm, while the samples L and F were obtained by a sieve with a size of 1.0 mm.

Table 4.4: Influent D, N, L and F - important ratios

Parameter	Unit	Influent D	Influent N	Influent L	Influent F
(CODt-CODs)/TSS	g/g	2.1	0.98	1.0	1.8
CODs/CODt	%	86.9	93.0	90.3	79.0
VFA-COD/CODs	%	16.9	11.7	14.6	9.1
VFA-COD/CODt	%	14.6	13.1	13.2	7.2
VSS/TSS	%	88.4	88.6	89.1	84.1
VS/DS	%	77.0	85.2	85.0	89.3
CODt/VS	g/g	2.0	1.8	2.0	2.0
CODt/$N_{soluble}$/$P_{soluble}$	-	100/0.62/0.27	100/0.84/0.22	100/0.78/0.21	100/0.52/0.10
VFA-COD/$N_{soluble}$/$P_{soluble}$	-	100/4.25/1.87	100/6.40/1.69	100/5.91/1.56	100/7.25/1.38

CODt – Total chemical oxygen demand; CODs – Chemical oxygen demand of the soluble compounds; TSS – Total suspended solids; VSS – Volatile suspended solids; DM – Dry matter; VM - Volatile matter; TKN – Kjeldahl nitrogen; P – Phosphorous; VFA – Volatile fatty acids; VFA-COD – Chemical oxygen demand content in volatile fatty acids.

The influents D, N, L and F contained a total of 54.5 g COD/L, 74.3 g COD/L, 79.6 g COD/L and 120.4 g COD/L respectively. The major part of this COD was for the four samples present as soluble compounds, as can be seen in Table 4.4. The pH of the wastewaters was below 4, which was expected as samples were taken after acidification. The degree of acidification reached in the acidification tank was 17% for sample D, 12% for sample N, 15% for sample L and 9% for sample F.

The concentrations of the suspended solids in influents D and N, after the 0.5 mm sieve, were 3.86 g/L and 5.27 g/L respectively. On the other hand, the concentrations of the suspended solids in influents L and F, after the 1.0 mm sieve, were 8.04 g/L and 14.17 g/L respectively.

The presence of nutrients (N and P) was evaluated based on the soluble concentrations, because the soluble compounds of N and P are more bio-available than the particulate compounds. The influent D contained 339 mg/L soluble TKN (nitrogen as ammonium and as soluble protein) and 149 mg/L soluble phosphorous. The influent N and L contained 622 mg/L soluble TKN and 164 mg/L soluble phosphorous. The influent F contained 630 mg/L soluble TKN and 120 mg/L soluble phosphorous.

The ratio COD/$N_{soluble}$/$P_{soluble}$ of the influent D was 100/0.62/0.27. For influent N and L, this ratio was 100/0.84/0.22 and for influent F it was 100/0.52/0.10. In case only the VFA are

considered as organic carbon source, the ratio VFA-COD/$N_{soluble}$/$P_{soluble}$ of influent D was 100/4.25/1.87. For influent N and L it was 100/6.40/1.69 and for influent F it was 100/7.25/1.38. In order to prevent nutrient shortages in an aerobic system, a COD/N/P ratio of 100/5/1 is advised. However, in the concept of producing reserve compounds from the influent, a shortage of a nutrient is desired to stimulate the bacteria to accumulate lipids.

Based on the above ratio's, a shortage of nitrogen and phosphorous was expected for all influents, in case all COD would be consumed. In case only VFA would be consumed in the quick scans, a nutrient shortage would probably not occur.

4.3.2. QUICK SCAN 1

Tables 4.5, 4.6 and 4.7 summarize the results obtained in the test performed with influent D.

Table 4.5: Determination of the presence of the feast and famine phase

Regime	Day	Time (h)	Reactor R_1		Reactor R_2	
			CODs (mg/L)	VFA (mg/L)	CODs (mg/L)	VFA (mg/L)
Feast	Day 2	1.5	1821	717	144	0
		2.5	1451	0	140	0
Famine		4	1234	0	125	0
		8	1160	0	108	0

R_1: Reactor submitted a high sludge loading rate of 1.21 g COD/(g VSS.d); R_2: Reactor submitted a low sludge loading rate of 0.19 g COD/(g VSS.d)

As can be seen in Table 4.5, reactor R_2 reached a famine phase after 2.5 hours because all VFA was consumed and there was no significant COD consumption between 2.5 and 8 hours (end of famine phase). In reactor R_1 no famine phase was reached as a significant amount of COD was converted between 2.5 and 8 hours of aeration.

Table 4.6: Results for sludge concentration in the growth reactors (the samples was taken at the end of the feast phase)

	Reactor R_1		Reactor R_2	
Day	Day 0	Day 3	Day 0	Day 3
TSS (g/L)	42.4	31.9	42.4	24.7
VSS (g/L)	16.5	19.1	16.5	10.9
VSS/TSS (%)	39	60	39	47

As it can be seen in Table 4.6 both reactors started with a high concentration of VSS, about 16.5 g/L. Sludge growth (increase in VSS concentration) occurred in reactor R_1 after 3 days of adaptation. However, in reactor R_2, the initial sludge concentration of 16.5 g VSS/L could not be maintained. Apparently, the sludge loading rate of 0.19 g COD/(g VSS.d) in reactor R_2 was too low to maintain the sludge and some sludge mineralization occurred. Nonetheless, the sludge concentration during the adaptation phase was of less importance, as this phase focusses on the selection of the desired organisms.

Table 4.7: Results for the sludge concentration in the filling up reactor (FUR) using the sample D as influent

Time	Units	Reactor R1		Reactor R2	
		0h	24h	0h	24h
Reactor volume	L	0.4	-	0,4	-
TSS	g/L	31.9	45.9*	24,7	35,9
VSS	g/L	19.1	29.1*	10,9	19,5
VSS/TSS	%	60	64	44	54
Influent added	g VFA-COD/(L.d)	3.0		2.9	
	g CODt/(L.d)	20.4		20.2	
Influent added	g TSS/(L.d)	2.3		2.3	
	g VSS/(L.d)	2.0		2.0	
TSS Yield	g TSS/ g $VFA\text{-}COD_{added}$	4.7		3.8	
VSS Yield	g VSS/ g $VFA\text{-}COD_{added}$	3.4		3.0	
TSS Yield	g TSS/ g $CODt_{added}$	0.69		0.56	
VSS Yield	g VSS/ g $CODt_{added}$	0.49		0.43	

*These values were adjusted to the initial volume and were corrected for the amount of TSS or VSS added with the influent.

Table 4.7 shows the results obtained for the fill-up test, using mixed liquor with 3 days of adaptation. In both reactors, the VSS content in the sludge increased, mainly in reactor R_2 from 44% to 54%. The VSS growth in both reactors was similar, about 8.6-10 g/L. Only the amount of the influent added during the test was monitored. Therefore, the sludge yields are based on the COD and VFA added. The sludge yields based on VFA were higher than 1. Consequently, the conclusion can be made that the activated sludge was able to consume also the COD present in the influents. Furthermore, the VSS yields obtained for both reactors were similar, about 0.43-0.49 g VSS/g $CODt_{added}$.

4.3.3. QUICK SCAN 2

Tables 4.8 and 4.9 summarize the results obtained in the test performed with influent N.

Table 4.8: Determination of the feast and famine regime in the batch reactors, feed with influent N

Regime	Day	Time (h)	Reactor R_1		Reactor R_2	
			CODs (mg/L)	VFA (mg/L)	CODs (mg/L)	VFA (mg/L)
Feast	Day 2	2	2531	510	105	0
		3	2448	470	105	0
Famine		7	2040	0	87.5	0

R_1 – Reactor submitted to a high sludge loading rate of 0.96 g COD/g VSS.d; R_2 – Reactor submitted to a low sludge loading rate of 0.15 g COD/g VSS.d

Overall, with the results shown in Table 4.8, it became evident that if the sludge was subjected to a low loading rate, 0.2 g COD/(g VSS.d), as in reactor R_2, the famine phase was reached at least after 2 hours of feeding because all VFA was consumed and there was no significant conversion of the soluble COD. The sludge of reactor R_1 was subjected to a high sludge loading rate of 1.14 g COD/(g VSS.d). It was observed that after 3 hours of aeration, 470 mg/L of VFA was still to consume. Further conversion of VFA and soluble COD occurred between 3 and 7 hours of aeration. It had to be concluded that no famine phase was reached after 2.5 hours in R_1.

Table 4.9: Results for the sludge concentration and lipid content in the growth reactors (at the end of the feast phase)

Parameter	Units	Reactor R_1				Reactor R_2			
Time	d	0	1	2	3	0	1	2	3
Influent flow rate	L/L.d	0.16				0.025			
Influent concentration	g COD/L	74.3				74.3			
	g VFA-COD/L	9.7				9.7			
Volumetric loading rate	g COD/(L.d)	11.89				**1.86**			
	g VFA-COD/(L.d)	**1.56**				0.24			
TSS (g/L)	g/L	26.6	15.7	21.5	21.1	26.6	14.8	19.9	15.6
VSS (g/L)	g/L	10.8	7.3	10.9	13.6	10.8	6.5	8.9	7.4
VSS/TSS (%)	%	40	47	51	64	40	44	45	47

In Table 4.9, it can be seen that the reactors were started with a high concentration of VSS, about 10.8 g/L. Sludge growth occurred in reactor R_1 after 3 days of adaptation. However, in

reactor R_2, the initial sludge concentration of 10.8 g VSS/L could not be maintained. Apparently, the sludge loading rate of 0.15 g COD/(g VSS.d) in reactor R_2 was too low to maintain the sludge and some sludge mineralization occurred. Nonetheless, the sludge concentration during the adaptation phase was of less importance, as this phase focusses on the selection of the desired organisms.

As previously described in section 4.2.2.2.2, several fill-up tests were performed with influent N to investigate the optimal duration of the adaptation phase. The activated sludge was sampled from the selector reactor and filled-up after 1 day and after 3 days of adaptation phase using influent N. The reactor R_2 was the only one where a feast and famine regime was obtained. So, it was expected that the best results during the fill-up phase would occur in R_2 where the organisms capable to store intracellular reserves as lipids were selected.

Tables 4.10 and 4.11 summarize the results obtained in the filling up phase using sludge collected from the selector reactor after 1 and 3 days of adaptation, respectively.

Table 4.10: Results for the sludge concentration and lipid content in the filling up phase using sample N as influent and SBR activated sludge after 1 day of adaption

Time	Units	Reactor R_1		Reactor R_2	
		0h	24h	0h	24h
Reactor volume	L	0.25	-	0,25	-
TSS	g/L	15.7	21.0*	14.8	24.5*
VSS	g/L	7.3	13.0*	6.5	14.3*
VSS/TSS	%	47	62	44	58
Influent flow rate	L/(L.d)	0.24		0.4	
Influent concentration	g COD/L	74.3			
	g VFA-COD/L	9.7			
Influent consumed	g VFA-COD/(L.d)	1.88		1.98	
	g CODt/(L.d)	12.5		16.7	
Influent added	g TSS/(L.d)	1.02		1.50	
	g VSS/(L.d)	0.90		1.33	
VSS Yield	**g VSS/g $CODt_{consumed}$**	**0.45**		**0.46**	

*These values were adjusted to the initial volume and were corrected for the amount of TSS and VSS added with the influent.

In the fill-up tests performed with sludge after 1 day of adaptation., the VSS content of the sludge increased substantially in both reactors, from 44-47% at the start to 58-62% in the end of

the experiment (Table 4.10). The VSS growth in reactor R_1 was about 5.7 g/L, consuming 12.5 g CODt/L. The VSS growth in reactor R_2 was about 7.8 g/L, consuming 16.7 g CODt/L. The VSS yield was similar in both reactors, about 0.45-0.46 g VSS/g COD consumed.

Table 4.11: Results for the sludge concentration and lipid content in the filling up phase using the sample N as influent and SBR activated sludge after 3 days of adaption

Time	Units	Reactor R_1		Reactor R_2	
		0h	24h	0h	24h
Reactor volume	L	0.25	-	0,25	-
TSS	g/L	21.1	26.3*	15.6	20.2*
VSS	g/L	13.6	18.0*	7.4	12.3*
VSS/TSS	%	64	68	47	61
Influent flow rate	L/(L.d)	0.20		0.24	
Influent concentration	g COD/L	74.3			
	g VFA-COD/L	9.7			
Influent consumed	g VFA-COD/(L.d)	1.6		0.5	
	g CODt/(L.d)	5.5		7.9	
Influent added	g TSS/(L.d)	0.9		1.0	
	g VSS/(L.d)	0.8		0.9	
VSS Yield	**g VSS/ g $CODt_{consumed}$**	**0.80**		**0.62**	

*Values were adjusted to the initial volume and were corrected for the amount of TSS or VSS added with the influent

In the fill-up tests performed with sludge after 3 days of adaptation, the VSS content of the sludge increased, mainly in reactor R_2 from 47% to 61% (Table 4.11). The VSS growth in reactor R_1 was about 4.4 g/L, consuming 5.5 g CODt/L. The VSS growth in reactor R_2 was about 4.9 g/L, consuming 7.9 g CODt/L. The VSS yields obtained for both reactors were above the sludge yield, 0.5 g VSS/g CODt, what is generally accepted as a maximal sludge yield. Those high VSS yields might indicate storage of intracellular reserves.

Comparing the two fill-up tests performed in this experiment (Quick Scan 2), based on the VSS yields, the lowest fill-up yields – not considering the adaptation phase – were obtained in a fill-up test after 1 day of adaptation. The applied sludge loading rate during that 1 day of adaptation did not influence the fill-up test. In both cases – high and low sludge loading rate during 1 day – a yield of 0.45 g VSS/g COD was obtained. This yield approximates the theoretical sludge yield

The highest fill-up yield in terms of VSS - not considering the adaptation phase - of 0.80 g VSS/g COD, was obtained after the SBR sludge was adapted to influent N during 3 days, at a high sludge loading rate (0.96 g COD/(g VSS.d)). In case the SBR sludge was adapted during 3 days at a low sludge loading rate (0.15 g COD/(g VSS.d)), a somewhat lower, but still good fill-up yield of 0.62 g VSS/g COD was realized. However, these sludge yields above 0.5 g VSS/g COD are very high and need repetition in case it would be considered to operate a full scale installation under those conditions.

4.3.4. QUICK SCAN 3

The aim of this experiment was to evaluate whether the activated sludge from the SBR could fill-up with a vegetable processing stream, without an adaptation phase. Therefore, this third quick scan consisted only in a single step – a fill up phase for 24 hours.

Table 4.12: Results for the sludge concentration, lipid content in the reactors D, N L and F

Time	Units	Reactor D		Reactor N		Reactor L		Reactor F	
		0h	24h	0h	24h	0h	24h	0h	24h
Volume	L	0.4	-	0.25	-	0.4	-	0.4	
TSS	g/L	17.5	21.9*	17.5	22.6*	40.2	44.4*	40.2	39.0*
VSS	g/L	7.5	11.9*	7.5	13.2*	17.2	22.3*	17.2	19.5*
VSS/TSS	%	43	55	43	58	43	50	43	50
Influent concentration	g COD/L	54.5		74.3		79.6		120.4	
	g VFA-COD/L	8.0		9.7		10.5		8.7	
Influent flow rate	L/L.d	0.48		0.52		0.35		0.35	
Influent consumed	g VFA-COD/(L.d)	1.47		1.79		1.10		1.40	
	g CODt/(L.d)	13.23		18.74		17.01		28.52	
Influent added	g TSS/(L.d)	1.38		2.08		2.30		4.61	
	g VSS/(L.d)	1.21		1.84		2.05		3.88	
	mg TKN/L	159		202		218		221	
	mg NH_4^+-N/L	76		53		62		71	
	mg PO_4^{3-}-P/L	77		53		57		42	
VSS Yield	g VSS/g $CODt_{rem}$	0.33		0.30		0.24		0.06	

*Values were adjusted to the initial volume and were corrected for the amount of TSS or VSS added with the influent

In Table 4.12 can be seen that in four reactors an increase in VSS content from 43% to 50-58% was observed. Over 24 hours of fill-up, the observed VSS growth in reactor D was about 4.4

g/L, in reactor N 5.7 g/L, in reactor L 5.1 g/L and in reactor F the observed VSS growth was about 2.3 g/L. Furthermore, in all reactors could be observed that the organisms consumed a high amount of COD: 13-29 g/L during 24 hours of fill-up. As described in section 4.4, all influents contained insufficient amounts of nutrients (N and P) for an optimal aerobic sludge growth. However, in the concept of producing reserve compounds from the influent, a shortage of a nutrient is desired to stimulate the organisms to accumulate lipid/PHA.

Observing the 3 fill-up tests reported on influent N (two in Quick Scan 2 and one in Quick Scan 3), it was found that the VSS yields based on VFA were higher than 1. Therefore, the conclusion can be made that the activated sludge converted more COD than only the VFA present in the influents. The lowest fill-up yields in terms of VSS – not considering the adaptation phase - were obtained in Quick Scan 3, without adaptation phase. However, the overall COD-to-VSS should be calculated taking into account both the adaptation and the fill-up phase. The overall balance for the quick scan on influent N is made in Table 13.

Table 4.13: Calculation of the global COD-to-VSS yields for influent N

		Units	Immediate fill-up	1 day + Fill-up		3 days + Fill-up	
				R_1	R_2	R_1	R_2
Sludge growth*	VSS	g/L	5.7	2.2	3.5	7.2	1.5
Input	CODt	g/L	18.7	24.4	18.3	52.4	29.3
VSS Yield	VSS / CODt	g/g	0.30	0.19	0.19	0.14	0.05

*Total sludge growth observed in adaptation + fill-up phase

In Table 4.13 can be seen that a growth of 5.7 g VSS/L was observed after 24 hours of filling-up without an adaptation phase. After 3 days of adaptation, at a low loading rate, plus 1 day of filling-up, the total increase in VSS concentration was 7.2 g VSS/L. The extra amount of VSS (7.2-5.7=1.5 g VSS/L) could not compensate, for the extra amount of COD that was pumped into the system to maintain the sludge during 3 days of adaptation. It is clear that the highest COD-to-VSS conversion yield was obtained in case the SBR sludge was immediately filled-up with the Laarakker influent sample N. Thus, the next Quick scans performed were aimed to improve the VSS yield.

Therefore the conclusion can be made that it is not necessary to have an adaptation phase to select the desired organisms, because the results obtained with this Quick Scan shows that the activated sludge used is already rich in organisms capable to store carbon sources.

4.3.5. QUICK SCAN 4

The aim of this experiment was to evaluate the influence of the pH set point to be used in the fill-up tests. This quick scan consisted in just a single step – a fill up phase for 24 hours at a different pH set point: 8.2. Table 4.14 summarizes the obtained results.

Table 4.14: Results for the sludge concentration in reactors L and F at pH 8.2

Time	Units	Reactor L		Reactor F	
		0h	24h	0h	24h
Volume	L	0.4	-	0,4	-
TSS	g/L	40.2	39.2*	40.2	40.8*
VSS	g/L	17.2	18.6*	17.2	20.0*
VSS/TSS	%	43	47	43	49
Influent concentration	g COD/L	79.6		120.4	
	g VFA-COD/L	10.5		8.7	
Influent flow rate	L/(L.d)	0.35		0.55	
COD removed	g CODt /(L.d)	9.8		20.8	
TSS added	g TSS/(L.d)	2.4		5.9	
VSS added	g VSS/(L.d)	2.1		5.0	
VSS Yield	g VSS/ g $CODt_{rem}$	0.07		0.07	

* These values were adjusted to the initial volume and were corrected for the amount of TSS and VSS added with the influent

The goal of this test was to increase the production of lipids by controlling the pH in the fill up phase at a higher set-point (8.2 instead of 7.2), in order that the cells would not grow and therefore just store carbon sources as lipids. Luísa Serafim *et al.* [153] found that a higher pH could be an extra stress factor for the bacteria to store intracellular reserves as lipids.

Comparing the results at pH controlled below 8.2, showed on Table 4.14, with the results obtained at pH controlled below 7.2 (Table 4.12), the conclusion can be made that the highest VSS yields were obtained at a pH set-point in the fill-up of 7.2.

4.3.6. QUICK SCAN 5

The aim of this experiment was to evaluate the minimal time necessary to the organisms to accumulate lipids. This quick scan consisted in only 1 step – fill up phase for 8 hours. Table 4.15 summarizes the obtained results.

Table 4.15: Results for the sludge concentration in reactors N and F

Time	Unit	Reactor N		Reactor F	
		0h	8h	0h	8h
Volume	L	0.40	0.47	0.40	0.48
TSS	g/L	32.2	34.2*	32.2	36.1*
VSS	g/L	13.6	17.2*	13.6	20.2*
VSS/TSS	%	42	50	42	56
Influent concentration	g COD/L	74.3		120.4	
	g VFA-COD/L	9.7		8.7	
Influent flow rate	L/(L.d)	0.25		0.25	
VFA-COD removed	g VFA-COD/(L.8h)	0.7		0.4	
COD removed	g CODt/(L.8h)	8.9		15.1	
P removed	mg P/(L.8h)	8		0.8	
N removed	mg TKN/(L.8h)	130		134	
TSS added	g TSS/(L.8h)	1.0		3.0	
VSS added	g VSS/(L.8h)	0.9		2.4	
VSS Yield	g VSS/g $CODt_{rem}$	0.40		0.43	

*These values were adjusted to the initial volume and were corrected for the amount of TSS and VSS added with the influent

In reactor F 0.80 mg P/L.8h and 15 g CODs/L.8h was removed. In reactor N 8 mg P/L.8h and 9 g CODs/L.8h was removed. The ratio P/CODs, in both reactors was too low to allow cell growth. So, with the ratio P/CODs the conclusion can be made that the cells on sludge could not grow because they did not take enough phosphorous from the medium.

In both reactors all nitrogen added was removed, about 130 mg TKN/(L.8h) In the reactor F the ratio N/CODs was about 0.87 g N/100 g CODs and in reactor N, the ratio was about 1.4 g N/100 g CODs. So, as the optimal ratio to allow growth of the cells is about 5 g N/100 g CODs, the conclusion can be made that the cells on sludge could not grow because did not take enough ammonia from the medium.

With the thermogravimetric analysis, it can be seen that the amount of VSS increased during 8h of the test. In reactor F, the VSS increased about 5.5 g VSS/L while in reactor N the VSS increased about 3.5 g VSS/L. As the lipid content is part of VSS, it may be concluded that the sludge during 8 hours were rich in lipid, because the amount of ammonia and the phosphorus removed was too short to occur growth of the biomass.

4.4. CONCLUSIONS

In the present study, the different media used to produce biomass enriched in storage compounds had slight differences in their composition due to the fact that samples were taken in different periods from a Dutch vegetable processing company. From the experimental work it can be concluded that the microbial source used consumed the VFA as well as the COD present in all the different vegetable waste streams tested. This way, a shortage of nutrients was observed, due to the low amounts of nitrogen and phosphate present in the complex media used. In the fill-up tests performed with influent N, using a mixed liquor subjected to an adaptation phase at high and a low loading rate, it was observed that the sludge yields were similar. Thus, an overall balance was labored, concluding that an adaptation phase, to select the organisms capable of accumulating reserve lipids is not necessary.

Creating another stress factor, besides the shortage of nutrients, to improve the biosynthesis and accumulation of reserve compounds, namely a higher pH, did not seem to work out, because the filling-up performed at pH set point of 8.2 gave a lower VSS yield than the filling-up at a pH set point of 7.2. This can be concluded after monitoring the VSS, because after all, the lipid content is a part of the VSS. If a good VSS yield can be obtained, subsequently the conclusion can be made that a good storage compound yield was obtained, since the medium used has shortage of nutrients, and thus there are no nutrients to allow growth of the biomass.

From the Quick Scans performed, the best sludge yields were obtained when using the influents N and F during 8 hours: 0.40-0.43 g VSS/g $CODt_{removed}$.

At this moment of the R&D, it was essential to characterize the biomass obtained in the mixed liquor, to evaluate which reserve compounds were being stored. Thus, some analytical methods should have been performed to identify and quantify the reserve compounds. These methods were inexistent in Avecom NV at that time. Despite of the methods developed during this thesis, mentioned in chapter 3, for quantifying PHA, no influent was available to repeat the test and no time to obtain more influent, to repeat the test and to perform the PHA analysis before my internship had come to an end. Nevertheless, a mind of an engineer has to be very critical about his work, certainly, other reserve compounds were produced besides PHAs due to low VFA content in the substrate used.

CHAPTER 5

BIOSYNTHESIS AND ACCUMULATION OF NEUTRAL LIPIDS FROM A COMPLEX HYDROCARBON-RICH WASTEWATER BY AN ENRICHED MIXED CULTURE

5.1. SUMMARY AND MOTIVATION

Many members of hydrocarbonoclastic bacteria are able to synthesize and accumulate neutral lipids in conditions of unbalanced nutrient availability, i.e. in the presence of an excess of carbon source and a shortage of an essential element for growth such as nitrogen or phosphorous. Therefore, this strict group of bacteria shows a high potential for application in biotechnological processes combining biological treatment of wastewaters contaminated with oil hydrocarbons and production of raw material for biofuel or oleochemical industry (production of detergents, surfactants, soaps, paints, cosmetics, etc.). Notwithstanding, no industry has yet implemented a similar process.

The aim of the study developed in this chapter was the optimization of biosynthesis and accumulation of reserve compounds from a wastewater rich in spent lubricants and motor oils. As inoculum, an enriched mixed culture of hydrocarbonoclastic bacteria, previously adapted to the substrate, was used. To accomplish the goal, the effect of different nitrogen concentrations, COD concentrations and cultivation time for PHAs, TAGs and WEs accumulation was evaluated. Those three factors, namely nitrogen, carbon and time, were selected for optimization of neutral lipids production in one experiment, using response surface methodology (RSM) according to the central composite design. In the assay, 15 different conditions were tested in duplicate and a quadruplicate central point. This study was carried out at the Center of Biological Engineering of Minho University, Braga, Portugal.

5.2. MATHERIALS AND METHODS

5.2.1. INOCULUM AND SUBSTRATES

5.2.1.1. Inoculum

The microbial source used was a mixed culture previously enriched in hydrocarbonoclastic bacteria capable of accumulating reserve compounds. This culture was originally collected from an activated sludge wastewater treatment unity located at ALSTOM Portugal, S.A., Maia, Portugal. This inoculum was selected since it was already adapted to the substrate used.

5.2.1.1. Substrates

In this part of the work, a complex carbon source was used as substrate: wastewater containing spent lubricants and motor oils collected from the same wastewater treatment unit mentioned above. The wastewater was decanted and the liquid part was autoclaved two times for 40 min at 120°C before being used. After this process, a chemical characterization was performed, evaluating some parameters, such as COD, total nitrogen (TN), nitrates, nitrites and TAN.

5.2.2. CULTURE PREPARATION

In order to obtain the amount of biomass needed for the designed experiment, 5 mL of the previously enriched mixed culture was inoculated in four Erlenmeyer of 250 mL with 45 mL of sterilized mineral salt (MS) medium (9 g/L $Na_2HPO_4.12H_2O$, 1.5 g/L KH_2PO_4, 0.2 g/L $MgSO_4.7H_2O$, 1.2 g/L Fe-NH_4.citrate, 20 g/L $CaCl_2$, 2g/L Hoagland solution and 0.5 g/L $NaHCO_3$). Afterwards, a certain amount of nitrogen source (NH_4Cl) and carbon source (wastewater rich in oil hydrocarbons) was added to the medium. The COD concentration was about 5 g/L while the nitrogen concentration was about 0.5 g/L. Using these conditions, cell growth is expected. Every 2-3 days the biomass was transferred to a new medium with the same conditions. These transfers occurred in asepsis and stopped after 14 days when the amount of biomass was enough to start the trial. The cell growth was followed by optical density at 600 nm.

5.2.3. EXPERIMENTAL DESIGN

The influence of nitrogen concentration, COD concentration and accumulation time on the PHAs, TAGs and WEs was evaluated, using response surface method (RSM) according to the central composite design. The use of a factorial design leads to optimized parameters with a minimum set of experiments and also to the possibility of obtaining a polynomial expression that describes the process yield [154]. Thus, it is possible to investigate the joint effect of two or more factors on a dependent variable. A factorial experiment design also facilitates the study of interactions, giving insights on the effects of different conditions tested, on the identifiable subgroups of subjects participating in the experiment [155].

The statistical software package Design-Expert® (Stat-Ease, Inc., Minneapolis, USA) was used to determine and analyze the data. The design class chosen to implement the experiments was a response surface with 32 assays including a quadruplicate central point. Estimation of the main effects of each individual variable, as well as their interaction effects were determined according to Eq. (5.1)

$$Y_i = \beta_0 + \sum \beta_i X_i + \sum \beta_i X_i^2 + \sum \beta_{ij} X_i X_j$$

(Eq. 5.1)

Where Y_i is the response variable, β_0 is the constant, β_i is the linear effect, β_{ii} is the quadratic effect, β_{ij} is the interactive effect and X_i is the coded factor level.

The composite design used was a Central Composite Circumscribed (CCC) design. CCC designs are the original form of the central composite design. The axial points are at a certain distance from the center based on the properties desired for the design and the number of factors in the design. Axial points establish new extremes for the low and high settings for all factors. Figure 5.1 illustrates a CCC design. These designs have circular, spherical, or hyperspherical symmetry and require 5 levels for each factor. Augmenting an existing factorial or resolution V fractional factorial design with star points can produce this design. Furthermore, CCC designs provide high quality predictions over the entire design space, but require factor settings outside the range of the factors in the factorial part [156].

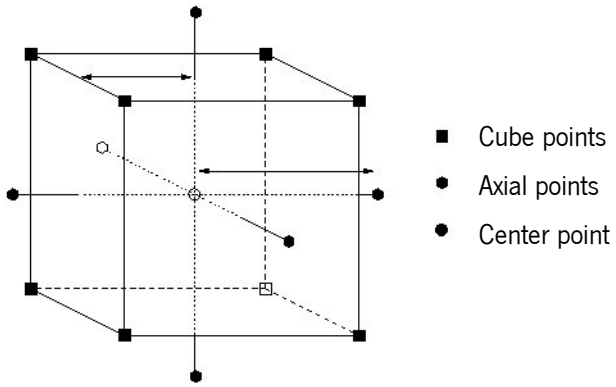

Figure 5.1: Example points of a Central Composite Circumscribed design with three input parameters [157].

Table 5.1 presents the limits used to determine the experimental design constituted with the 32 assays mentioned before.

Table 5.1: Design summary using RSM according to the central composite circumscribed design

	Coded Values / Parameters	-1	1
Actual values	A: COD (g/L)	14.05	25.95
	B: N (g/L)	0.056	0.16
	C: t (h)	29.03	78.97

Analysis of variance (ANOVA) was applied to identify the statistically significant terms. Significance of regression coefficients was determined with a confidence level of 95%. The coded values of the variables investigated are shown in Table 5.3. The medium used was the same as describe above.

Table 5.2: Coded values of central composite experimental design matrix defining total nitrogen (N) concentration, COD concentration and time (t)

Run	A:COD (g/L)	B: N (g/L)	C: t (h)	Run	A: COD (g/L)	B: N (g/L)	C: t (h)
15	0	0	-1.682	**10**	0	1.682	0
22	0	0	-1.682	**26**	0	1.682	0
2	-1	-1	-1	**12**	0	0	0
24	-1	-1	-1	**25**	0	0	0
6	1	-1	-1	**16**	0	0	0
5	1	-1	-1	**21**	0	0	0
7	-1	1	-1	**28**	-1	-1	1
18	-1	1	-1	**13**	-1	-1	1

Table 5.3: Coded values of central composite experimental design matrix defining total nitrogen (N) concentration, COD concentration and time (t)

Run	A:COD (g/L)	B: N (g/L)	C: t (h)	Run	A: COD (g/L)	B: N (g/L)	C: t (h)
29	1	1	-1	**11**	1	-1	1
27	1	1	-1	**17**	1	-1	1
20	-1.682	0	0	**23**	-1	1	1
9	-1.682	0	0	**14**	-1	1	1
31	1.682	0	0	**3**	1	1	1
8	1.682	0	0	**1**	1	1	1
32	0	-1.682	0	**30**	0	0	1.682
19	0	-1.682	0	**4**	0	0	1.682

5.2.4. ANALYTICAL PROCEDURES

COD, TN, nitrates, nitrites and TAN were estimated using the commercial kits LCK 914, LCK 338, LCK 340, LCK 341, LCK 303 (Hach-Lange®, Germany). All analyses were done in triplicate, following the procedures recommended by the commercial kits.

5.2.4.1. Extraction and analysis of storage compounds

Samples were centrifuged for 10 min at 10 000 g in a SIGMA 4k15 centrifuge. The supernatant was removed and the pellet obtained containing biomass was resuspended in a sterilized solution of 0.9% NaCl. Afterwards, the samples were centrifuged using the same conditions and the pellet was kept at -20°C and then lyophilized.

5.2.4.2. PHA analysis

To estimate the PHA content in the biomass a procedure developed by Vieira [158] was used. In particular, two PHA were analyzed: the poly-3-hydroxyvalyrate (PHV) and PHB.

First, a calibration curve was prepared using PHB and PHV standards. 9 different weights in a range of 0.4 to 2.7 mg from those polymers were used. Afterwards, between 10 and 23 mg of lyophilized biomass was used to perform this analysis using digestion tubes. Besides the standards and samples, a control was also carried out, in which an empty digestion tube was used before the addition of the reagents.

To the digestion tubes it was added 1.5 mL of internal standard, benzoic acid in dichloromethane (1 mg/mL) and 1.5 mL of 1-propanol-HCl (75:25 v/v). After closing and

agitation of the digestion tubes using a vortex, they were placed in the digester at 100°C during 3.5 hours. After cooling down, the volume of the digestion tubes was transfer to glass flasks of 10 mL. 2.0 mL were used to wash the digestion tubes and then placed in the respective glass flask. Then, the glass flasks were sealed and agitated during 1 min using vortex. This way, both phases, the organic phase and aqueous phase, were in contact, optimizing the extraction of PHAs.

To allow the separation of phases, the flasks were inverted during 30 min. After this period, the organic phase (the heaviest phase) was transferred to vials of 1.5 mL where Na_2SO_4 was added to ensure dehydration of this phase. Then, the resultant volume was transferred to another vial of 1.5 mL and stored at -20°C.

All samples, standards and control were analyzed by gas chromatography with flame ionization detector (GC-FID). The GC Varian 3800, Varian Inc, USA, equipment was used with a TR-WAX (Teknokroma, Spain) column with a dimensions of 30m x 0.32 mm x 0.25 mm. The injector and detector temperature was 220°C and 250°C, respectively. The column temperature was 50°C during 2 min, then increased at 15°C/min until 225°C and was kept at 225°C during 5 minutes. The air flux used was 250 mL/min, the hydrogen flux was 30 mL/min, while the helium (drag gas) flux was 1 mL/min (50 kPa of pressure in column) and the nitrogen flux was 30 mL/min. Finally, the injection volume used was 2 µL.

5.2.4.3. Extraction and fractionation of neutral lipids

The intracellular lipids were extracted from the biomass with chloroform/methanol (2:1, v/v) in a proportion of 2 mL of solvent per 10 mg of lyophilized biomass [159]. The lyophilized biomass was weighted in glass flasks. Afterwards, the glass flasks were sealed and agitated during 2 hours at room temperature. Then, the cellular debris was rejected by filtration using glass wool. The resultant volume was kept in another glass flask until all solvent had been evaporated.

Lipidic extracts were fractionated using a solid phase technique based on compound class. Elution of different lipid compound classes was conducted on columns of SPE silica (SiOH) Chromabond 1000-mg, 6 mL (Macherey-Nagel, Düren, Germany), operating three solvent systems: pure n-hexane, 94/6 (v/v) n-hexane/diethyl ether and 85/15/2 (v/v/v) n-hexane/diethyl ether/acetic acid. Optimization of the separation was achieved by performing thin layer chromatography (TLC). Silica columns were previously placed into a Supelco® vacuum

Manifold system in the fume hood. Then, lipids extract were loaded, in 1 mL of *n*-hexane, into the silica column, which was pre-conditioned with 5 mL of *n*-hexane. Afterwards the sample glass flask was rinsed two times with 0.5 mL of *n*-hexane and added to the column. When the entire sample was in the column, an elution was performed using 3.7 mL of 94/6 (v/v) n-hexane/diethyl ether using vacuum. Just before expose the column packing to air, the vacuum was turned off, the collection flask was changed and it was added 13.5 mL of 94/6 (v/v) *n*-hexane/diethyl ether. With vacuum on, 15 mL of 85/15/2 (v/v/v) n-hexane/diethyl ether/acetic acid was added into the column, obtaining a second fraction. These two fractions were collected in glass flasks, previously weighted. In the first fraction were eluted the hydrocarbons remained from wastewater used and in the second fraction neutral lipids were collected. Therefore, quantification by weight of neutral lipids was carried out when all solvent had been evaporated. This method was optimized based on solid phase extraction described Revellame *et al* [160].

Neutral lipid extracts obtained from solid phase separation technique were reconstituted in the minimum volume of chloroform to be applied on TLC plates. These fractions were spotted on 10 x 10 cm glass DC-Fertigplatten plates, precoated with 0.25 mm silica gel 60 with fluorescent indicator UV_{254} (Macherey-Nagel, Germany). These qualitative analyses were performed using the solvent system hexane/diethyl ether/acetic acid (80:20:1). Sample applications were conducted using Micropippetes, Blaubrand® intra MARK with a size of 5 µL (Wertheim, Germany) with a Micro-pipettierhelger Classic (Wertheim, Germany). Neutral lipids were visualized on plates by staining with iodine vapor. Olive oil, oleic acid, and oleyl oleate were used as reference substances for TAGs, fatty acids and WEs, respectively [19].

5.2.4.4. Total hydrocarbons

Hydrocarbons present in the wastewater were analyzed and quantified by GC-FID. First of all, a calibration curve was performed based on a robust method for the determination of mineral oil in water samples [161]. A mineral oil standard mixture type A and B for EN 14039 and ISO 16703, Fluka® was used as an initial standard stock solution. Calibrations solutions 0.2, 0.4, 0.6, 0.8, 1.0 and 1.5 mg/mL were prepared by dilution in *n*-hexane.

A sample of the wastewater was cleaned up using a column Sep-Pak Florisil 6cc (Waters, USA) into a Supelco® vacuum Manifold system in the fume hood. 820 µL of wastewater was loaded, into the column, which was preconditioned with 5 mL of *n*-hexane. Then, 5 mL of dichloromethane was loaded into the column, three times to make sure that all traces are eluted.

The resultant volume was kept in a glass flask until all solvent had been evaporated. After this period, a dilution of 1000 times, using *n*-hexane, was performed to be analyzed by GC-FID.

The GC Varian 4000, Varian Inc, USA, equipment was used a VF-1MS column with dimensions of 15 m x 0.25 mm. The injector and detector temperature was at 250°C and 315°C, respectively. The column temperature was 60°C during 1 min, then increased at 8°C/min until 270°C and was kept at 270°C during 17.75 minutes. The air flux used was 250 mL/min, the hydrogen flux was 30 mL/min, while the helium (drag gas) flux was 1 mL/min and the nitrogen flux was 30 mL/min. Finally, the injection volume used was 2 µL using an inlet split/splitness.

5.3. RESULTS AND DISCUSSION

5.3.1. WASTEWATER CHARACTERIZATION

The wastewater used as carbon source in this experiment was characterized and the results of the parameters evaluated are shown in Table 5.3. The organic nitrogen content of the wastewater was calculated and the results are shown in the same Table.

Table 5.4: Characteristics from the wastewater collected from the wastewater treatment unity at ALSTOM Portugal, S.A., Maia, Portugal

Parameter	Unit	Sample from: May
COD	g/L	307 ± 14
Total hydrocarbons	g/L	541 ± 8
TN	mg/L	172 ± 18
Nitrates	mg/L	93.8 ± 8.8
Nitrites	mg/L	0
TAN	mg/L	1.3 ± 0.4
Organic nitrogen [1]	mg/L	76.6 ± 8.9
pH		5.5

1 – Organic nitrogen = TN - (Nitrates + Nitrites) organic nitrogen was determined by the difference between the total nitrogen and the inorganic nitrogen (Nitrates and Nitrites).

High levels of COD and total hydrocarbons were detected in the analyzed wastewater, namely 307 ± 14 and 541 ± 8 g/L, respectively. On the opposite, the nutrient content, nitrogen and

phosphorus, was quite low, specially comparing to the amount of COD, being a limiting factor for the growth of the microorganisms if it would be used as substrate. In addition, the wastewater showed a slight level of acidity. However, this fact did not affect the use of wastewater as substrate in the experiment.

The determination of total organic carbon (TOC) was quite inaccurate (high variation in the measured values, using Varian TOC analyzer), limiting the direct calculation of C/N ratio. To obtain an estimate of this value, it was considered a ratio between COD and total organic carbon of about 4, based on the theoretical values calculated for saturated hydrocarbons with the general formula of C_nH_{2n+2}.

5.3.2. PROCESS OPTIMIZATION

5.3.2.1. OPTIMIZATION OF PHA ACCUMULATION

According to the experimental design, a culture enriched in hydrocarbonoclastic bacteria was cultivated in a medium that contained different concentrations of a wastewater rich in oil hydrocarbons (carbon source), with varied concentrations of NH_4Cl (nitrogen source) and during different times of incubation. This way, RSM, according to the central composite design, was used to optimize the C/N ratio and incubation time that leads to the highest values of PHA content. The central composite design consisted in 32 experiments.

RSM is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes in which a response of interest is influenced by several variables and the objective is to optimize this response [162]. The analysis developed a model by fitting the experimental data in a generalized smooth curve, from which a specific predicted response could be calculated. Thus, the response surface analysis established a relation between the variable and response more efficiently than the traditional design [163, 164].

The experimental central composite design matrix is presented in Table 5.3. In this case only the siginificant regression having P-values < 0.05, indicating that they are significant, were considered into the model. Therefore, the siginificant model terms identified by the software used were [N], [COD]x[N], $[COD]^2$ and t^2.

The software derived model equation was:

$$Y = 0.067 - (5.19 \times 10^{-3}) \times [A]' - 7.209 \times 10^{-3} \times [B]' + 3.718 \times 10^{-3} \times C' + 0.014 \times [A]' \times [B]' - 7.723 \times 10^{-3} \times [A]'^2 - 0.015 \times C'^2$$

(Eq. 5.2)

where Y represents PHA content (g/g), [A]', [B]' and [C]' are the transformed forms of the independent variables according to:

$$Z' = \frac{X_i - \frac{X^+ + X^-}{2}}{\frac{X^+ + X^-}{2}}$$

(Eq. 5.3)

Where X_i is the original (untransformed) value of the variables, and X^+ and X^- are the upper and lower limit values of the variables.

In equation 5.2, [A] represents COD concentration (g/L), [B] represents nitrogen concentration (g/L) and C represents time (h).

Equation 5.2 is a quadratic polynomial expression which indicates that those quadratic terms of COD concentration and time show a negative non-linear effect on the accumulation of PHA as can be verified in Figure 5.2. Moreover, it is possible to verify that the linear coefficient of nitrogen concentration has a negative effect. This comes back to what was expected, because for lower concentrations of nitrogen the biomass was subjected to limiting conditions of growth leading to the production and storage of PHA. It must be highlighted that the interaction [COD]x[N] is considered a significant term, indicating that those variables are not independent of each other. However, the interaction between [COD]xt and [N]xt was found to be insignificant (P-values > 0.05).

Central composite circumscribed design is illustrated in a 3-dimensional graph of the calculated response surface for the siginificant model terms identified as shown in Figure 5.2.

In this work, as can be seen in Figure 5.2, highest PHA accumulations were found for the lowest COD concentration as well as for the lowest nitrogen concentration, during the different times experimented. Observing the results, it is possible to see that for the same carbon (estimated from the COD) to nitrogen different results are obtained. An example of this fact is highlighted in Figure 5.2, where for two points having the same ratio C/N (ratio C/N = 300), the point with lower COD and nitrogen exhibited higher PHA content than the point with higher COD and nitrogen concentrations. This can be explained by the fact that with higher concentrations of

COD and N, probably the biomass was not in limiting conditions of nitrogen, and was subsequently growing instead of producing storage compounds such as PHA.

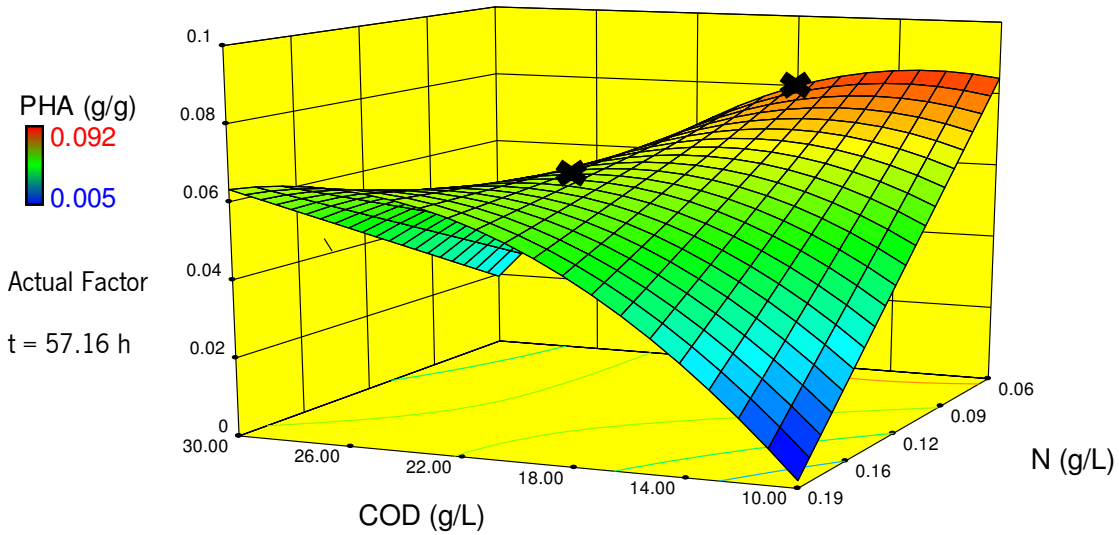

Figure 5.2: Response surface plot of the effect of COD and N concentrations on maximum PHA content at t = 57.16 hours (curves: calculated values from experimental data (31 values) by the statistical software package "Design-Expert 7.0, Stat-Ese, Inc.").

During the experimental procedure, it was noticed that when weighing the lyophilized biomass that it was sticky and, in some cases, traces of wastewater were found. Consequently, the results for PHA content have been underestimated (the bests results obtained were not even 10% g PHA/g biomass, Table 5.5). As the wastewater consists mostly of oil and lubricants, which are non-soluble in water, the process of biomass separation from the medium was quite difficult. After centrifugation, a settle down biomass forming a pellet would be expected, after which it would be easy to separate the biomass. But, part of the biomass formed an upper layer together with the wastewater that was not consumed by the biomass. Probably, in this layer of biomass, organisms enriched with lipids are found, hindering the separation of the phases. As a result, part of the non-degraded substrate was mixed with the biomass and contributed for the solids of the analyzed dry biomass material.

Table 5.5: Experimental data obtained from the assay defined by Central composite experimental design matrix

Run	COD (g/L)	N (g/L)	t (h)	C/N	% PHB (g/g)	% PHV (g/g)	% PHA (g/g)
15	20	0.1058	12	47	0.45%	0.05%	0.50%
22	20	0.1058	12	47	0.71%	0.07%	0.78%
2	14	0.0557	29	63	8.02%	1.13%	9.15%
24	14	0.0557	29	63	3.26%	0.34%	3.60%
6	26	0.0557	29	116	2.54%	0.25%	2.78%
5	26	0.0557	29	116	3.57%	0.38%	3.95%
7	14	0.1559	29	23	3.14%	0.47%	3.60%
18	14	0.1559	29	23	2.04%	0.22%	2.25%
29	26	0.1559	29	42	4.33%	0.60%	4.93%
27	26	0.1559	29	42	5.43%	0.92%	6.35%
20	10	0.1058	54	24	5.45%	0.67%	6.11%
9	10	0.1058	54	24	4.45%	0.54%	4.98%
31	30	0.1058	54	71	3.03%	0.37%	3.40%
8	30	0.1058	54	71	2.78%	0.36%	3.14%
32[1]	20	0.0216	54	231	1.19%	0.12%	1.31%
21	20	0.0216	54	231	7.75%	0.82%	8.57%
10	20	0.1900	54	26	4.52%	0.68%	5.20%
26	20	0.1900	54	26	5.65%	0.71%	6.36%
12	20	0.1058	54	47	4.71%	0.49%	5.19%
25	20	0.1058	54	47	4.97%	0.66%	5.62%
16	20	0.1058	54	47	7.24%	0.78%	8.02%
21	20	0.1058	54	47	5.53%	0.73%	6.26%
28	14	0.0557	79	63	2.03%	0.23%	2.26%
13	14	0.0557	79	63	2.57%	0.34%	2.91%
11	26	0.0557	79	116	3.23%	0.37%	3.60%
17	26	0.0557	79	116	2.87%	0.31%	3.17%
23	14	0.1559	79	23	5.39%	0.72%	6.11%
14	14	0.1559	79	23	8.15%	1.02%	9.17%
3	26	0.1559	79	42	2.90%	0.37%	3.27%
1	26	0.1559	79	42	3.97%	0.51%	4.49%
30	20	0.1058	96	47	3.53%	0.45%	3.97%
4	20	0.1058	96	47	3.80%	0.52%	4.32%

[1] - The experimental data obtained from run 15 was not considered in the analysis (statistically identified as an outlier)

The total PHA in the samples was calculated as the sum of the measurement of PHB and PHV. Experimentally, for the assay where the highest amount of PHA was obtained, 14 g COD/L,

0.16 g N/L and 79 hours was used. In these conditions the PHA content was 9.2% in the biomass. However, from the response surface plot obtained (Figure 5.2) it is possible to conclude that the optimal values for concentrations of COD, nitrogen and time are 14.05 g/L, 0.06 g/L and 57 hours respectively. In these conditions the PHA content would be 8.6% in the biomass. The model obtained points to use lower COD and nitrogen concentrations compared to those used in this approach. Despite the fact that the time for maximum PHA content had been found, it is recommended to test a range around that time in a future approach because for lower concentrations of COD and nitrogen, the optimal time production can be different.

Table 5.6: Content of PHA in the inoculum used in the experiment

Inoculum	% PHB (g/g)	% PHV (g/g)	% PHA (g/g)
(a)	2.04 %	0.13 %	2.16 %
(b)	1.52 %	0.12 %	1.64 %

In Table 5.5 can be observed that two replicates of the inoculum, used in the experiment, were analyzed. The average PHA content in the biomass, at the beginning of the experiment, was about 1.90 %. Therefore, a conclusion can be made that the biomass was able to increase the amount of PHA 5 times, when it was subjected to the lowest COD and N concentrations tested in the experiment.

Literature has shown that the carbon to nitrogen ratio (C/N) of the media is one of the key factors to achieve optimal PHA accumulation [68]. Table 5.7 summarizes a few studies that investigated the importance of C/N ratio, where it is possible to observe the diversity of optimal C/N ratio found; depending on the source of inoculum and the source of substrate. There are no studies in the literature using spent oil hydrocarbons as substrate. In this study, it could be seen that C/N ratio had a significant effect on PHA percentage accumulated in the cells using a complex oil hydrocarbon-based wastewater. The optimum C/N ratio found was around 60 (g/g), which is within the range between 9 and 100 (g/g) described in literature. However, the analysis made in this study suggested that higher ratios can be applied to obtain maximum yield from the tested wastewater.

Table 5.7: Summary of a few detailed studies about C/N ratio in PHA accumulation

PHA content	Optimal C/N ratio	Unit	Substrate	Type of culture	Reference
60 %	19	mol/mol	Oleic acid	Pure - *Pseudomonas aeruginosa*	Hong C. *et al.* [165]
19 %	50	g/g	Defined	Mixed – Activated sludge	Basak, B. *et al.* [166]
29 %	25	g/g	Defined	Mixed – Activated sludge	Mokhtarani, N. *et al.* [167]
40 %	9	g/g	Defined	Pure - *Thermus thermophilus*	Papaneophytoy, C. and D. kyriakidis [162]
27 %	100	g/g	Complex	Mixed – Activated sludge	Wang, Y.J. *et al.* [168]
49 %	60	g/g	Synthetic	Mixed – Activated sludge	Hong, C. *et al.* [164]

5.2.4.4. Analysis of neutral lipids accumulation

Besides the accumulation of PHAs, accumulation of other reserve compounds, such as wax ester and triacylglycerols, was also analyzed. These reserve compounds can have interesting applications due to their higher caloric value, low grade of oxidation and their relative compactness [24, 32, 33]. These can be interesting characteristics in view of oleochemical and biofuel industries. Valorization of residues as wastewater together with production of potentially high-added value lipids, could become a process economically viable and simultaneously beneficial for the environment. WEs, TAGs and FAs acids can be considered as neutral lipids.

To analyze the neutral lipids accumulated in the biomass, it was necessary to separate the oil hydrocarbons from the extract (using silica columns) since they were found to interfere with the TLC development. Figure 5.3 shows the TLC analysis of the first fraction eluted from the silica columns where it is possible to observe that the separation of the oil hydrocarbons and neutral lipids was successful and no neutral lipids were eluted with it. Without this separation, the TLC analysis of neutral lipids accumulated in the biomass, which can be observed in Figure 5.4, would not have been possible.

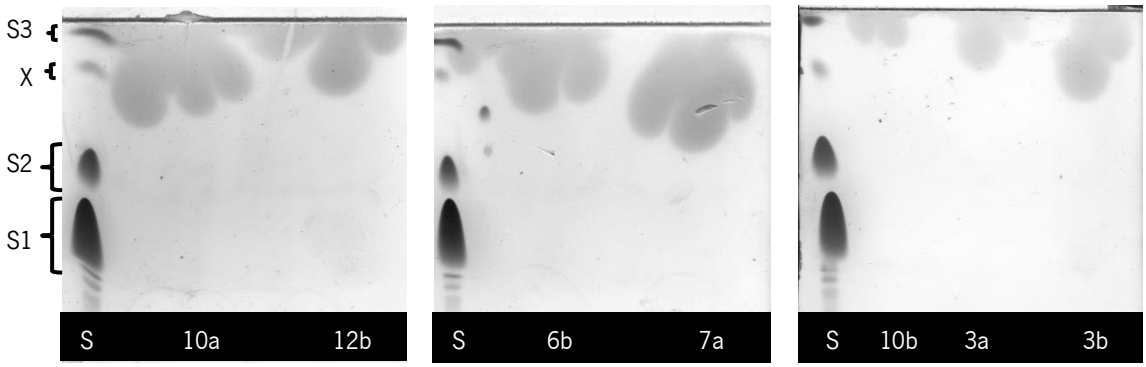

Figure 5.3: TLC analysis of hydrocarbons separated in the first fraction separated from the lipidic extracts. S – standard mix: S1 – oleic acid (standard of FA), S2 – olive oil (standard of TAGs) and S3 - oley oleate (standard of WEs). X - unknown. Identity of the samples is given in Table 5.7.

Looking at Figure 5.4, overall it is possible to observe a wide range of neutral lipids. Many of them were not possible to identify, because the bands of the standards used did not correspond to the bands observed in the samples analyzed. The inoculum, sample 0a and 0b, was also analyzed. FAs, TAGs and WEs could be detected in sample 0a, in very low intensity, while in sample 0b only FAs were possible to detect. FAs were present in all samples. Particularly, in samples 2a, 3b, 4b, the bands had a high intensity, showing that its presence can be quite high. The biomasses of these samples have a cultivation time of 29h in common.

Looking at the presence of TAGs in Figure 5.4, it can be quite hard to identify which fragments can be considered TAGs. However, its presence was detected in most of the conditions studied, except in samples 5b, 13d and 14b. Looking at their replicates (5a, 13a, 13b, 13c and 14a) we can conclude that those conditions (26 g COD/L, 0.1559 g N/L and 29 h; 20 g COD/L, 0.1058 g N/L and 54 h; 26 g COD/L, 0.1559 g N/L and 79 h) are not suitable for the accumulation of TAGs, because their bands in the TAGs region had a very low intensity. The biomass of those samples was submitted to higher concentrations of COD and N than the average used in the experimental design. This is in accordance with what was found in the optimization of PHA, i.e. that the higher concentrations of COD and N used in experimental design are not suitable for accumulation of reserve compounds.

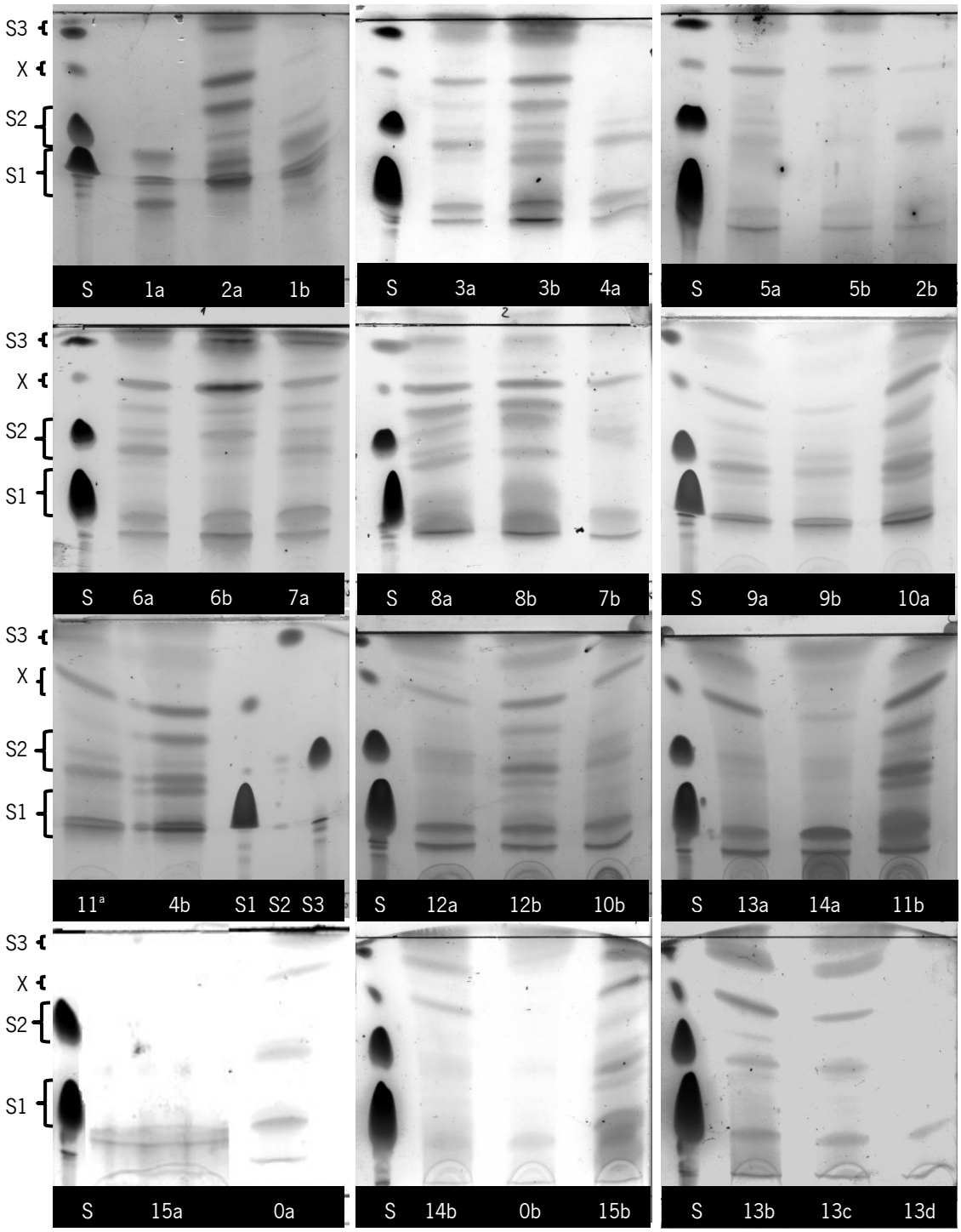

Figure 5.4: TLC analysis of neutral lipids accumulated in the sludge under the tested conditions. S – standard mix: S1 – oleic acid (standard of FA), S2 – olive oil (standard of TAGs) and S3 - oley oleate (standard of WEs). X - unknown. Identity of the samples is given in Table 5.7.

Regarding the presence of WEs, when looking at Figure 5.4 the conclusion can be made that its accumulation also occurred in a few conditions tested, besides other accumulated reserve compounds. In addition, samples such as 1a, 1b, 2b, 4a, 4b, 7b, 9a, 9b, 13d and 15a, did not have presence of WEs. On the other hand, TLC analysis of sample 6b showed a band with a high

intensity of WEs. In particular, this sample had another band with high intensity that corresponds to an unknown lipid existing in the oleic acid standard (S1), thought to be resulting from a contamination. This unknown lipid probably belongs to WEs with a different polarity than the standard WEs used in the current study. Furthermore, this unknown lipid was present in several samples, except in samples 1a, 1b, 2b, 4a, 9b and 15a. These samples match with samples where the biomasses did not accumulate wax esters. This suggests that in those conditions, accumulation of WEs is not possible. In future work, identification of this unknown lipid would be interesting because its accumulation occurred in several samples analyzed by TLC.

Table 5.8: Experimental conditions applied for each sample analyzed by TLC

Sample	COD (g/L)	N (g/L)	t (h)	Sample	COD (g/L)	N (g/L)	t (h)
1a	20	0.1058	12	**10a**	14	0.0557	79
2a	14	0.1559	29	**11a**	20	0.0216	54
1b	20	0.1058	12	**4b**	14	0.0557	29
3a	26	0.0557	29	**12a**	26	0.0557	79
3b	26	0.0557	29	**12b**	26	0.0557	79
4a	14	0.0557	29	**10b**	14	0.0557	79
5a	26	0.1559	29	**13a**	20	0.1058	54
5b	26	0.1559	29	**14a**	26	0.1559	79
2b	14	0.1559	29	**11b**	20	0.0216	54
6a	20	0.1900	54	**15a**	20	0.1058	96
6b	20	0.1900	54	**0a**	inoculum		
7a	10	0.1058	54	**14b**	26	0.1559	79
8a	30	0.1058	54	**0b**	inoculum		
8b	30	0.1058	54	**15b**	20	0.1058	96
7b	10	0.1058	54	**13b**	20	0.1058	54
9a	14	0.1559	79	**13c**	20	0.1058	54
9b	14	0.1559	79	**13d**	20	0.1058	54

The qualitative analysis performed allowed to verify that several conditions tested are suitable for the accumulation of WEs, TAGs and FAs. However, further identification and quantification of the WEs and TAGs synthesized would be of much interest, allowing the application of the response surface methodology and, therefore, find the conditions that optimize its accumulation.

Nevertheless, the results obtained by TLC are very encouraging, showing the wide range of neutral lipids that can be obtained. Besides that, another encouraging fact is that in the TLC analysis the inoculum exhibited poor content in neutral lipids, showing that the culture used could accumulate several neutral lipids when subjected to the tested conditions, using a wastewater contaminated with motor oils and lubricants as substrate.

5.4. CONCLUSIONS

The work developed in this chapter allowed to evaluate the effect of different nitrogen concentration, COD concentration and incubation time in the biosynthesis and accumulation of bacterial reserve compounds from a wastewater containing spent lubricants and motor oils. Overall, the biomass used, a culture enriched in hydrocarbonoclastic bacteria, was able to accumulate three different types of reserve compounds, namely polyhydroxyalkanoates, wax esters and triacylglycerols.

According to the experimental design, the highest values of PHA content were obtained for lower concentrations of COD and nitrogen. The optimal value achieved from the statistical software used was obtained for the conditions of 14 g COD/L, 0.06 g N/L with a cultivation time of 57 hours. In these conditions the PHA content in the biomass is about 8.6%.

From the qualitative analysis performed to evaluate the presence of neutral lipids, it was possible to observe a wide range of neutral lipids in most of the conditions tested. Besides accumulation of PHAs, the culture used was able to synthesize other reserve compounds such as WEs and TAGs. Therefore, a quantification of the neutral lipids identified is recommended, to obtain values for the final composition of the biomass in terms of reserve compounds. Moreover, this Chapter shows relevant results for the use of spent oil hydrocarbons, by using a mixed culture that can handle removal of hydrocarbons with the production of compounds that can afterwards be used by the biofuel or oleochemical industry. Use of this kind of substrate, with low or even negative cost, combined with mixed cultures, where there is no need to run a sterile process and with subsequently less energy demands, can lead to a large acceptance in industry, being an economically and environmentally sustainable process.

CHAPTER 6

GENERAL CONCLUSIONS AND FUTURE WORK

6.1. GENERAL CONCLUSIONS AND FINAL REMARKS

This research project aimed to study the production of reserve compounds such as polyhydroxyalkanoates, triacylglycerols and wax esters using mixed bacterial cultures. This study can be divided in three main parts where some objectives were defined. The first part included the production and optimization at pilot scale of a novel product: biomass enriched in polyhydroxyalkanoates, for application trials. This main goal was accomplished with complete success, with an installation of a complete set-up to produce enriched sludge in PHA at pilot scale. To reach the main goal proposed, many challenges had to be overcome. The first challenge to overcome was to have a mixed bacterial culture enriched with about 40% of PHA, after fermentation. The second challenge to overcome was to stabilize the cells, i.e. to prevent conversion of the intracellular PHA and stored during fermentation. The third challenge to overcome was improving the settling characteristics to get a quick and efficient thickening and drying of the sludge. A last but not least challenge was to determine the most suitable way to store the final product. Besides that, the study went further than what was expected in the beginning. Analytical methods were developed for quantification of the novel product, which were inexistent at Avecom NV.

The second part included optimization and production at lab scale of reserve compounds by fermentation of organic side streams. From the experimental work developed, it can be concluded that an adaptation phase, to select the organisms capable of accumulating reserve lipids is not recommended because high enough VSS yields in the fill-up tests with non-adapted biomass were obtained. From batch experiments performed, the best sludge yields were obtained when influents N (74 g CODt/L, 0.16 g N/L, 0.16 g P/L and 9.7 g VFA-COD/L) and F (120 g CODt/L, 0.20 g N/L, 0.12 g P/L and 8.7 g VFA-COD/L) were supplied during 8 hours fill-in: 0.40-0.43 g VSS/g $CODt_{removed}$ at a high COD removal in combination with a very low nitrogen and phosphate uptake.

In the third part of the work, a detailed study was performed regarding the biosynthesis and accumulation of reserve compounds from a wastewater contaminated with lubricants and oil motors. By performing the experimental work, according to an experimental design, using a RSM, three different reserve compounds were identified in the biomass in most of the conditions tested.

The reserve compounds found in the biomass were PHAs, WEs and TAGs. Neutral lipids, WEs and TAGs, were identified by TLC analysis while PHAs were quantified by analyzing the PHBs and PHVs present in the biomass using GC-FID. Applying RSM, optimal PHA accumulation would be obtained for the lowest nitrogen and COD concentrations used in this study. Therefore, the optimal values for PHA accumulation are obtained for 14 g COD/L, 0.06 g N/L and a cultivation time of 57 hours.

Modern society depends on fossil resources stored within the earth. People most often link these resources to energy. Coal and natural gas are used to create electricity, while petroleum is refined to be used in fuels. Oil and natural gas are used to synthesize and produce plastics. These plastics are used to manufacture several benefits and have become an integral part of modern life. As society moves towards a sustainable economy, it is essential that new methods are developed for producing both fuels and chemicals. Biotechnology can be applied to convert renewable resources into chemical products [57]. Therefore, I hope that my results will prove to be useful in the field of reserve compounds research and contribute to the development of a sustainable chemical industry.

6.2. SUGGESTIONS FOR FUTURE WORK

Perhaps, one of the most interesting things in research is the further needs of study identified alongside the work. In this scope, and in particular for the first part of the work, the production of a novel product, the analysis developed during the internship at Avecom NV should be confirmed and evaluated for other products that contain PHAs, to consolidate the knowledge about the method. However, they showed to be reproducible and reliable for biomass enriched with PHA. Furthermore, samples of the final product should be characterized by institutions with known background in this area. It would be interesting to know which PHAs are present in the final product. This issue can be important in research, in view of a possible application, where this product would be included and, subsequently, helping in those possible application trials.

Moreover, an economical study about the possibility to extract PHAs from biomass, after the fermentation step using propylene carbonate as solvent, would be interesting. As previously

pointed out, propylene carbonate has a high boiling point, about 240°C, preventing evaporation to the environment and allowing its reusability for several sets of purification. This could decrease the solvent consumption, being economically profitable. This solvent is widely used in many applications including cosmetic industry.

Regarding the second part of the work, to develop and continue the work performed, a repetition of the batch experiment where the best yields were obtained is recommended, to perform a characterization of the biomass obtained from the mixed liquor in order to evaluate which reserve compounds were being stored. This is a crucial step before thinking on an up-scale or another approach.

Concerning the third and last part of the work, for future work a quantification of the different neutral lipids identified by TLC analysis would be of value, to also implement a response surface methodology, as was applied for PHA accumulation. Consequently, an optimization of the process would be possible, as well as an evaluation of its potential to be implemented in industry, even though, there is still much work to be done. Besides a quantification of neutral lipids, further work can be performed. For example, monitoring the consumption of hydrocarbons, using a total hydrocarbons method developed during this dissertation, as a monitoring of growth of the biomass during cultivation time makes it possible to calculate the biomass yield. Subsequently, by monitoring the content of each reserve compound identified into the biomass, it would be possible to calculate a yield for each reserve compound. Therefore, the option to do an up-scale would be evaluated according to the yields calculated.

Moreover, an evaluation of the microbial composition dynamics in the assays, performed using 16S RNA gene base molecular methods, such as Denaturing Gradient Gel Electrophoresis (DGGE), would also be interesting, to see if some conditions, where a high accumulation of certain kind of reserve compound was verified, can be linked with the predominance certain lipid accumulating microorganisms. In literature, lipid export has already been reported in some organisms. Therefore, this phenomenon can be evaluated, by analyzing the presence of reserve compounds in the medium culture.

REFERENCES

REFERENCES

1. Wackett, L.P., *Microbial-based motor fuels: science and technology.* Microbial Biotechnology, 2008. **1**(3): p. 211-225.
2. Grant, L., *When will the oil run out?* Science, 2005. **309**(5731): p. 52-+.
3. Rojo, F., *Biofuels from microbes: a comprehensive view.* Microbial Biotechnology, 2008. **1**(3): p. 208-210.
4. Antoni, D., V.V. Zverlov, and W.H. Schwarz, *Biofuels from microbes.* Applied Microbiology and Biotechnology, 2007. **77**(1): p. 23-35.
5. Rottig, A., L. Wenning, D. Broker, and A. Steinbuchel, *Fatty acid alkyl esters: perspectives for production of alternative biofuels.* Applied Microbiology and Biotechnology, 2010. **85**(6): p. 1713-1733.
6. Li, Y., Z. Zhao, and F. Bai, *High-density cultivation of oleaginous yeast Rhodosporidium toruloides Y4 in fed-batch culture.* Enzyme and Microbial Technology, 2007. **41**(3): p. 312-317.
7. Angerbauer, C., M. Siebenhofer, M. Mittelbach, and G.M. Guebitz, *Conversion of sewage sludge into lipids by Lipomyces starkeyi for biodiesel production.* Bioresource Technology, 2008. **99**(8): p. 3051-3056.
8. Zhao, X., X. Kong, Y. Hua, B. Feng, and Z. Zhao, *Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast Lipomyces starkeyi.* European Journal of Lipid Science and Technology, 2008. **110**(5): p. 405-412.
9. Zhu, L.Y., M.H. Zong, and H. Wu, *Efficient lipid production with Trichosporon fermentans and its use for biodiesel preparation.* Bioresource Technology, 2008. **99**(16): p. 7881-7885.
10. Lois, E., *Definition of biodiesel.* Fuel, 2007. **86**(7-8): p. 1212-1213.
11. Adamczak, M., U.T. Bornscheuer, and W. Bednarski, *The application of biotechnological methods for the synthesis of biodiesel.* European Journal of Lipid Science and Technology, 2009. **111**(8): p. 800-813.
12. Alvarez, H.M., *Biology of Rhodococcus.* Springer Verlag, 2010.
13. Li, Q., W. Du, and D.H. Liu, *Perspectives of microbial oils for biodiesel production.* Applied Microbiology and Biotechnology, 2008. **80**(5): p. 749-756.

14. Fakas, S., S. Papanikolaou, M. Galiotou-Panayotou, M. Komaitis, and G. Aggelis, *Organic nitrogen of tomato waste hydrolysate enhances glucose uptake and lipid accumulation in Cunninghamella echinulata.* Journal of Applied Microbiology, 2008. **105**(4): p. 1062-1070.

15. Papanikolaou, S. and G. Aggelis, *Biotechnological valorization of biodiesel derived glycerol waste through production of single cell oil and citric acid by Yarrowia lipolytica.* Lipid Technology, 2009. **21**(4): p. 83-87.

16. Papanikolaou, S. and G. Aggelis, *Yarrowia lipolytica: A model microorganism used for the production of tailor-made lipids.* European Journal of Lipid Science and Technology, 2010. **112**(6): p. 639-654.

17. Papanikolaou, S. and G. Aggelis, *Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production.* European Journal of Lipid Science and Technology, 2011. **113**(8): p. 1031-1051.

18. Shah, S.N., B.K. Sharma, and B.R. Moser, *Preparation of Biofuel Using Acetylatation of Jojoba Fatty Alcohols and Assessment as a Blend Component in Ultralow Sulfur Diesel Fuel.* Energy & Fuels, 2010. **24**: p. 3189-3194.

19. Manilla-Perez, E., A.B. Lange, H. Luftmann, H. Robenek, and A. Steinbuchel, *Neutral lipid production in Alcanivorax borkumensis SK2 and other marine hydrocarbonoclastic bacteria.* European Journal of Lipid Science and Technology, 2011. **113**(1): p. 8-17.

20. Wältermann, M. and A. Steinbüchel, *Wax Ester and Triacylglycerol Inclusions*, in *Inclusions in Prokaryotes*, J. Shively, Editor 2006, Springer Berlin Heidelberg. p. 137-166.

21. Alvarez, H.M. and A. Steinbuchel, *Triacylglycerols in prokaryotic microorganisms.* Applied Microbiology and Biotechnology, 2002. **60**(4): p. 367-376.

22. Brandl, H., R.A. Gross, R.W. Lenz, and R.C. Fuller, *Plastics from bacteria and for bacteria: poly(beta-hydroxyalkanoates) as natural, biocompatible, and biodegradable polyesters.* Advances in biochemical engineering/biotechnology, 1990. **41**: p. 77-93.

23. Lee, S.Y. and H.N. Chang, *Production of poly(hydroxyalkanoic acid).* Advances in biochemical engineering/biotechnology, 1995. **52**: p. 27-58.

24. Lenz, R.W. and R.H. Marchessault, *Bacterial polyesters: Biosynthesis, biodegradable plastics and biotechnology.* Biomacromolecules, 2005. **6**(1): p. 1-8.

25. Hanggi, U.J., *REQUIREMENTS ON BACTERIAL POLYESTERS AS FUTURE SUBSTITUTE FOR CONVENTIONAL PLASTICS FOR CONSUMER-GOODS.* Fems Microbiology Reviews, 1995. **16**(2-3): p. 213-220.

26. Steinbuchel, A. and B. Fuchtenbusch, *Bacterial and other biological systems for polyester production.* Trends in Biotechnology, 1998. **16**(10): p. 419-427.

27. Angelova, N. and D. Hunkeler, *Rationalizing the design of polymeric biomaterials.* Trends in Biotechnology, 1999. **17**(10): p. 409-421.

28. Waltermann, M. and A. Steinbuchel, *Neutral lipid bodies in prokaryotes: Recent insights into structure, formation, and relationship to eukaryotic lipid depots.* Journal of Bacteriology, 2005. **187**(11): p. 3607-3619.

29. Olukoshi, E.R. and N.M. Packter, *IMPORTANCE OF STORED TRIACYLGLYCEROLS IN STREPTOMYCES - POSSIBLE CARBON SOURCE FAR ANTIBIOTICS.* Microbiology-Uk, 1994. **140**: p. 931-943.

30. Alvarez, H.M., R. Kalscheuer, and A. Steinbuchel, *Accumulation and mobilization of storage lipids by Rhodococcus opacus PD630 and Rhodococcus ruber NCIMB 40126.* Applied Microbiology and Biotechnology, 2000. **54**(2): p. 218-223.

31. Manilla-Perez, E., A.B. Lange, S. Hetzler, and A. Steinbuchel, *Occurrence, production, and export of lipophilic compounds by hydrocarbonoclastic marine bacteria and their potential use to produce bulk chemicals from hydrocarbons.* Applied Microbiology and Biotechnology, 2010. **86**(6): p. 1693-1706.

32. Murphy, D.J., *STRUCTURE, FUNCTION AND BIOGENESIS OF STORAGE LIPID BODIES AND OLEOSINS IN PLANTS.* Progress in Lipid Research, 1993. **32**(3): p. 247-280.

33. Alvarez, H.M., F. Mayer, D. Fabritius, and A. Steinbuchel, *Formation of intracytoplasmic lipid inclusions by Rhodococcus opacus strain PD630.* Archives of Microbiology, 1996. **165**(6): p. 377-386.

34. Goutx, M., M. Acquaviva, and J.C. Bertrand, *CELLULAR AND EXTRACELLULAR CARBOHYDRATES AND LIPIDS FROM MARINE-BACTERIA DURING GROWTH ON SOLUBLE SUBSTRATES AND HYDROCARBONS.* Marine Ecology Progress Series, 1990. **61**(3): p. 291-296.

35. Bredemeier, R., R. Hulsch, J.O. Metzger, and L. Berthe-Corti, *Submersed culture production of extracellular wax esters by the marine bacterium Fundibacter jadensis.* Marine Biotechnology, 2003. **5**(6): p. 579-583.

36. Solaiman, D.K.Y., R.D. Ashby, and T.A. Foglia, *Medium-chain-length poly(beta-hydroxyalkanoate) synthesis from triacylglycerols by Pseudomonas saccharophila.* Current Microbiology, 1999. **38**(3): p. 151-154.

37. Solaiman, D.K.Y., R.D. Ashby, and T.A. Foglia, *Production of polyhydroxyalkanoates from intact triacylglycerols by genetically engineered Pseudomonas.* Applied Microbiology and Biotechnology, 2001. **56**(5-6): p. 664-669.

38. Ishige, T., A. Tani, K. Takabe, K. Kawasaki, Y. Sakai, and N. Kato, *Wax ester production from n-alkanes by Acinetobacter sp strain M-1: Ultrastructure of cellular inclusions and role of acyl coenzyme A reductase.* Applied and Environmental Microbiology, 2002. **68**(3): p. 1192-1195.

39. Singer, M.E., S.M. Tyler, and W.R. Finnerty, *GROWTH OF ACINETOBACTER SP STRAIN HO1-N ON NORMAL-HEXADECANOL - PHYSIOLOGICAL AND ULTRASTRUCTURAL CHARACTERISTICS.* Journal of Bacteriology, 1985. **162**(1): p. 162-169.

40. Miwa, T.K., J.A. Rothfus, and E. Dimitroff, *EXTREME-PRESSURE LUBRICANT TESTS ON JOJOBA AND SPERM WHALE OILS.* Journal of the American Oil Chemists Society, 1979. **56**(8): p. 765-770.

41. Wisniak, J., *Jojoba oil and derivatives.* Progress in the chemistry of fats and other lipids, 1977. **15**(3): p. 167-218.

42. Dewitt, S., J.L. Ervin, D. Howesorchison, D. Dalietos, S.L. Neidleman, and J. Geigert, *SATURATED AND UNSATURATED WAX ESTERS PRODUCED BY ACINETOBACTER SP-HO1-N GROWN ON C16-C20 N-ALKANES.* Journal of the American Oil Chemists Society, 1982. **59**(2): p. 69-74.

43. Stoveken, T., R. Kalscheuer, U. Malkus, R. Reichelt, and A. Steinbuchel, *The wax ester synthase/acyl coenzyme A : diacylglycerol acyltransferase from Acinetobacter sp strain ADP1: Characterization of a novel type of acyltransferase.* Journal of Bacteriology, 2005. **187**(4): p. 1369-1376.

44. Barney, B.M., R.L. Mann, and J.M. Ohlert, *Identification of a Residue Affecting Fatty Alcohol Selectivity in Wax Ester Synthase.* Applied and Environmental Microbiology, 2013. **79**(1): p. 396-399.

45. Barney, B.M., B.D. Wahlen, E. Garner, J. Wei, and L.C. Seefeldt, *Differences in Substrate Specificities of Five Bacterial Wax Ester Synthases.* Applied and Environmental Microbiology, 2012. **78**(16): p. 5734-5745.

46. Chen, G.-Q., *Plastics from bacteria: Natural Functions and Applications*. Alexander Steinbüchel ed2010, Department of Biological Sciences and Biotechnology: Springer Heidelberg Dordrecht

47. Parlane, N.A., *Development and use of polyhydroxybutyrate biopolyester as particulate vaccine beads*, 2012, Massey University: Manawatu, New Zealand.

48. Repaske, R. and A.C. Repaske, *QUANTITATIVE REQUIREMENTS FOR EXPONENTIAL-GROWTH OF ALCALIGENES-EUTROPHUS.* Applied and Environmental Microbiology, 1976. **32**(4): p. 585-591.

49. Ward, A.C., B.I. Rowley, and E.A. Dawes, *EFFECT OF OXYGEN AND NITROGEN LIMITATION ON POLY-BETA-HYDROXYBUTYRATE BIOSYNTHESIS IN AMMONIUM-GROWN AZOTOBACTER-BEIJERINCKII.* Journal of General Microbiology, 1977. **102**(SEP): p. 61-68.

50. Philip, S., T. Keshavarz, and I. Roy, *Polyhydroxyalkanoates: biodegradable polymers with a range of applications.* Journal of Chemical Technology and Biotechnology, 2007. **82**(3): p. 233-247.

51. Anderson, A.J. and E.A. Dawes, *OCCURRENCE, METABOLISM, METABOLIC ROLE, AND INDUSTRIAL USES OF BACTERIAL POLYHYDROXYALKANOATES.* Microbiological Reviews, 1990. **54**(4): p. 450-472.

52. Sanguanchaipaiwong, V.B. and F.o.S.U. Biomolecular Sciences, *Biosynthesis and characterisation ofpolyhydroxyalkanoate based natural-synthetichybrid copolymers*, 2006.

53. Byrom, D., *Biomaterials : novel materials from biological sources*1991, New York: Stockton Press.

54. Doi, Y., *Plastics from microbes. Microbial synthesis of polymers and polymer precursors. Edited by D. P. Mobley, Hanser, Munich 1994, hardcover.* 1995: p. 5-33.

55. Khanna, S. and A.K. Srivastava, *Recent advances in microbial polyhydroxyalkanoates.* Process Biochemistry, 2005. **40**(2): p. 607-619.

56. Sudesh, K., H. Abe, and Y. Doi, *Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters.* Progress in Polymer Science, 2000. **25**(10): p. 1503-1555.

57. Budde, C.F., *Production of polyhydroxyalkanoate copolymers from plant oil*, in *Department of Chemical Engineering* 2010, Massachusetts institute of technology: USA.

58. Lemoigne, M., *The production of acid and beta-oxybutyric lactides by Bacillus mesesentericus vulgatus.* Comptes Rendus Des Seances De La Societe De Biologie Et De Ses Filiales, 1926. **94**: p. 1291-1292.

59. Zinn, M., B. Witholt, and T. Egli, *Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate.* Advanced Drug Delivery Reviews, 2001. **53**(1): p. 5-21.

60. Eggink, G., P. Dewaard, and G.N.M. Huijberts, *FORMATION OF NOVEL POLY(HYDROXYALKANOATES) FROM LONG-CHAIN FATTY-ACIDS.* Canadian Journal of Microbiology, 1995. **41**: p. 14-21.

61. Lee, S.Y., *Bacterial polyhydroxyalkanoates.* Biotechnology and Bioengineering, 1996. **49**(1): p. 1-14.

62. Doi, Y., *Polyhydroxyalkanoates.* Handbook of Biodegradable Polymers, 1997. **7**: p. 79-86.

63. Hrabak, O., *INDUSTRIAL-PRODUCTION OF POLY-BETA-HYDROXYBUTYRATE.* Fems Microbiology Reviews, 1992. **9**(2-4): p. 251-255.

64. Mousavioun, P., *Properties of lignin and polyhydroxybutyrate blends*, in *Chemistry*2011, Queensland University of Technology: Queensland, Australia.

65. Steinbuchel, A., *Polyhydroxyalkanoic acids*. Biomaterials, ed. D. Byrom1991, Basingstoke: MacMilan.

66. Lee, S.Y. and J.I. Choi, *Effect of fermentation performance on the economics of poly(3-hydroxybutyrate) production by Alcaligenes latus.* Polymer Degradation and Stability, 1998. **59**(1-3): p. 387-393.

67. Madison, L.L. and G.W. Huisman, *Metabolic engineering of poly(3-hydroxyalkanoates): From DNA to plastic.* Microbiology and Molecular Biology Reviews, 1999. **63**(1): p. 21-+.

68. Grothe, E., M. Moo-Young, and Y. Chisti, *Fermentation optimization for the production of poly(beta-hydroxybutyric acid) microbial thermoplastic.* Enzyme and Microbial Technology, 1999. **25**(1-2): p. 132-141.

69. Choi, J.I. and S.Y. Lee, *Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation.* Bioprocess Engineering, 1997. **17**(6): p. 335-342.

70. Lee, S.Y., *Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria.* Trends in Biotechnology, 1996. **14**(11): p. 431-438.

71. Reddy, C.S.K., R. Ghai, Rashmi, and V.C. Kalia, *Polyhydroxyalkanoates: an overview.* Bioresource Technology, 2003. **87**(2): p. 137-146.

72. De Schryver, P., *Poly-β-hydroxybutyrate as a microbial agent in aquaculture*, 2010, University of Ghent: Gent, Belgium.

73. Lee, S.Y., J.I. Choi, and H.H. Wong, *Recent advances in polyhydroxyalkanoate production by bacterial fermentation: mini-review.* International Journal of Biological Macromolecules, 1999. **25**(1-3): p. 31-36.

74. Serafim, L.S., P.C. Lemos, M.G.E. Albuquerque, and M.A.M. Reis, *Strategies for PHA production by mixed cultures and renewable waste materials.* Applied Microbiology and Biotechnology, 2008. **81**(4): p. 615-628.

75. Wallen, L.L. and Rohwedde.Wk, *POLY-BETA-HYDROXYALKANOATE FROM ACTIVATED-SLUDGE.* Environmental Science & Technology, 1974. **8**(6): p. 576-579.

76. Morgan-Sagastume, F., A. Karlsson, P. Johansson, S. Pratt, N. Boon, P. Lant, and A. Werker, *Production of polyhydroxyalkanoates in open, mixed cultures from a waste sludge stream containing high levels of soluble organics, nitrogen and phosphorus.* Water Research, 2010. **44**(18): p. 5196-5211.

77. Mudliar, S.N., A.N. Vaidya, M.S. Kumar, S. Dahikar, and T. Chakrabarti, *Techno-economic evaluation of PHB production from activated sludge.* Clean Technologies and Environmental Policy, 2008. **10**(3): p. 255-262.

78. Cai, M.M., H. Chua, Q.L. Zhao, S.N. Shirley, and J. Ren, *Optimal production of polyhydroxyalkanoates (PHA) in activated sludge fed by volatile fatty acids (VFAs) generated from alkaline excess sludge fermentation.* Bioresource Technology, 2009. **100**(3): p. 1399-1405.

79. Dionisi, D., M. Beccari, S. Di Gregorio, M. Majone, M.P. Papini, and G. Vallini, *Storage of biodegradable polymers by an enriched microbial community in a sequencing batch reactor operated at high organic load rate.* Journal of Chemical Technology and Biotechnology, 2005. **80**(11): p. 1306-1318.

80. Chang, H.F., W.C. Chang, and C.Y. Tsai, *Synthesis of poly(3-hydroxybutyrate/3-hydroxyvalerate) from propionate-fed activated sludge under various carbon sources.* Bioresource Technology, 2012. **113**: p. 51-57.

81. Dias, J.M.L., P.C. Lemos, L.S. Serafim, C. Oliveira, M. Eiroa, M.G.E. Albuquerque, A.M. Ramos, R. Oliveira, and M.A.M. Reis, *Recent advances in polyhydroxyalkanoate*

production by mixed aerobic cultures: From the substrate to the final product. Macromolecular Bioscience, 2006. **6**(11): p. 885-906.

82. Salehizadeh, H. and M.C.M. Van Loosdrecht, *Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance.* Biotechnology Advances, 2004. **22**(3): p. 261-279.

83. Lemos, P.C., L.S. Serafim, and M.A.M. Reis, *Polyhydroxyalkanoates production by activated sludge in a SBR using acetate and propionate as carbon sources.* Water Science and Technology, 2004. **50**(10): p. 189-194.

84. Castilho, L.R., D.A. Mitchel, and D.M.G. Freire, *Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation.* Bioresource Technology, 2009. **100**(23): p. 5996-6009.

85. Johnson, K., R. Kleerebezem, and M.C.M. van Loosdrecht, *Influence of the C/N ratio on the performance of polyhydroxybutyrate (PHB) producing sequencing batch reactors at short SRTs.* Water Research, 2010. **44**(7): p. 2141-2152.

86. Chua, A.S.M., H. Takabatake, H. Satoh, and T. Mino, *Production of polyhydroxyalkanoates (PHA) by activated sludge treating municipal wastewater: effect of pH, sludge retention time (SRT), and acetate concentration in influent.* Water Research, 2003. **37**(15): p. 3602-3611.

87. Rhu, D.H., W.H. Lee, J.Y. Kim, and E. Choi, *Polyhydroxyalkanoate (PHA) production from waste.* Water Science and Technology, 2003. **48**(8): p. 221-228.

88. Holmes, P.A., *APPLICATIONS OF PHB - A MICROBIALLY PRODUCED BIODEGRADABLE THERMOPLASTIC.* Physics in Technology, 1985. **16**(1): p. 32-36.

89. Luengo, J.M., B. Garcia, A. Sandoval, G. Naharro, and E.R. Olivera, *Bioplastics from microorganisms.* Current Opinion in Microbiology, 2003. **6**(3): p. 251-260.

90. Chen, G.-Q., *A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry.* Chemical Society Reviews, 2009. **38**(8): p. 2434-2446.

91. Chen, G.Q. and Q. Wu, *Microbial production and applications of chiral hydroxyalkanoates.* Applied Microbiology and Biotechnology, 2005. **67**(5): p. 592-599.

92. Chen, G.Q. and Q. Wu, *The application of polyhydroxyalkanoates as tissue engineering materials.* Biomaterials, 2005. **26**(33): p. 6565-6578.

93. Massieu, L., M.L. Haces, T. Montiel, and K. Hernandez-Fonseca, *Acetoacetate protects hippocampal neurons against glutamate-mediated neuronal damage during glycolysis inhibition.* Neuroscience, 2003. **120**(2): p. 365-378.

94. Zou, X.-H., H.-M. Li, S. Wang, M. Leski, Y.-C. Yao, X.-D. Yang, Q.-J. Huang, and G.-Q. Chen, *The effect of 3-hydroxybutyrate methyl ester on learning and memory in mice.* Biomaterials, 2009. **30**(8): p. 1532-1541.

95. Kashiwaya, Y., T. Takeshima, N. Mori, K. Nakashima, K. Clarke, and R.L. Veech, *D-beta-hydroxybutyrate protects neurons in models of Alzheimer's and Parkinson's disease.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(10): p. 5440-5444.

96. Williams, S.F., D.P. Martin, D.M. Horowitz, and O.P. Peoples, *PHA applications: Addressing the price performance issue I. Tissue engineering.* International Journal of Biological Macromolecules, 1999. **25**(1-3): p. 111-121.

97. Muller, S., T. Bley, and W. Babel, *Adaptive responses of Ralstonia eutropha to feast and famine conditions analysed by flow cytometry.* Journal of Biotechnology, 1999. **75**(2-3): p. 81-97.

98. Zhao, Y.H., H.M. Li, L.F. Qin, H.H. Wang, and G.-Q. Chen, *Disruption of the polyhydroxyalkanoate synthase gene in Aeromonas hydrophila reduces its survival ability under stress conditions.* Fems Microbiology Letters, 2007. **276**(1): p. 34-41.

99. Zhang, X.J., R.C. Luo, Z. Wang, Y. Deng, and G.Q. Chen, *Application of (R)-3-Hydroxyalkanoate Methyl Esters Derived from Microbial Polyhydroxyalkanoates as Novel Biofuels.* Biomacromolecules, 2009. **10**(4): p. 707-711.

100. Stuart, E.S., A. Tehrani, H.E. Valentin, D. Dennis, R.W. Lenz, and R.C. Fuller, *Protein organization on the PHA inclusion cytoplasmic boundary.* Journal of Biotechnology, 1998. **64**(2-3): p. 137-144.

101. Beun, J.J., F. Paletta, M.C.M. Van Loosdrecht, and J.J. Heijnen, *Stoichiometry and kinetics of poly-beta-hydroxybutyrate metabolism in aerobic, slow growing, activated sludge cultures.* Biotechnology and Bioengineering, 2000. **67**(4): p. 379-389.

102. Majone, M., P. Massanisso, A. Carucci, K. Lindrea, and V. Tandoi, *Influence of storage on kinetic selection to control aerobic filamentous bulking.* Water Science and Technology, 1996. **34**(5-6): p. 223-232.

103. Serafim, L.S., P.C. Lemos, R. Oliveira, and M.A.M. Reis, *Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions.* Biotechnology and Bioengineering, 2004. **87**(2): p. 145-160.

104. Suzuki, T., A.A. Tsygankov, J. Miyake, Y. Tokiwa, and Y. Asada, *ACCUMULATION OF POLY-(HYDROXYBUTYRATE) BY A NONSULFUR PHOTOSYNTHETIC BACTERIUM, RHODOBACTER-SPHAEROIDES RV AT DIFFERENT PH.* Biotechnology Letters, 1995. **17**(4): p. 395-400.

105. Mengmeng, C., C. Hong, Z. Qingliang, S.N. Shirley, and R. Jie, *Optimal production of polyhydroxyalkanoates (PHA) in activated sludge fed by volatile fatty acids (VFAs) generated from alkaline excess sludge fermentation.* Bioresource Technology, 2009. **100**(3): p. 1399-1405.

106. Nonato, R.V., P.E. Mantelatto, and C.E.V. Rossell, *Integrated production of biodegradable plastic, sugar and ethanol.* Applied Microbiology and Biotechnology, 2001. **57**(1-2): p. 1-5.

107. Shi, H.D., M. Shiraishi, and K. Shimizu, *Metabolic flux analysis for biosynthesis of poly(beta-hydroxybutyric acid) in Alcaligenes eutrophus from various carbon sources.* Journal of Fermentation and Bioengineering, 1997. **84**(6): p. 579-587.

108. Jacquel, N., C.W. Lo, Y.H. Wei, H.S. Wu, and S.S. Wang, *Isolation and purification of bacterial poly (3-hydroxyalkanoates).* Biochemical Engineering Journal, 2008. **39**(1): p. 15-27.

109. Ramsay, J.A., E. Berger, R. Voyer, C. Chavarie, and B.A. Ramsay, *EXTRACTION OF POLY-3-HYDROXYBUTYRATE USING CHLORINATED SOLVENTS.* Biotechnology Techniques, 1994. **8**(8): p. 589-594.

110. Kunasundari, B. and K. Sudesh, *Isolation and recovery of microbial polyhydroxyalkanoates.* Express Polymer Letters, 2011. **5**(7): p. 620-634.

111. Lafferty, R.M.H.E., *Use of cyclic carbonic acid esters as solvents for poly-(β-hydroxybutyric acid)*, in *Patent 414074*, U.S., Editor 1979: USA.

112. Yu, J. and L.X.L. Chen, *Cost-effective recovery and purification of polyhydroxyalkanoates by selective dissolution of cell mass.* Biotechnology Progress, 2006. **22**(2): p. 547-553.

113. Tamer, I.M., M. Moo-Young, and Y. Chisti, *Disruption of Alcaligenes latus for recovery of poly(beta-hydroxybutyric acid): Comparison of high-pressure homogenization, bead*

milling, and chemically induced lysis. Industrial & Engineering Chemistry Research, 1998. **37**(5): p. 1807-1814.

114. McChalicher, C.W.J., F. Srienc, and D.P. Rouse, *Solubility and Degradation of Polyhydroxyalkanoate Biopolymers in Propylene Carbonate.* Aiche Journal, 2010. **56**(6): p. 1616-1625.
115. Fiorese, M.L., F. Freitas, J. Pais, A.M. Ramos, G.M.F. de Aragao, and M.A.M. Reis, *Recovery of polyhydroxybutyrate (PHB) from Cupriavidus necator biomass by solvent extraction with 1,2-propylene carbonate.* Engineering in Life Sciences, 2009. **9**(6): p. 454-461.
116. Valappil, S.P., S.K. Misra, A.R. Boccaccini, T. Keshavarz, C. Bucke, and I. Roy, *Large-scale production and efficient recovery of PHB with desirable material properties, from the newly characterised Bacillus cereus SPV.* Journal of Biotechnology, 2007. **132**(3): p. 251-258.
117. Narasimhan K., C.A.C., Gibson M. S., Welling S. J., *Process for the solvent-based extraction of polyhydroxyalkanoates from biomass*, in *Patent 7378266*, U.S., Editor 2008: USA.
118. Wampfler, B., T. Ramsauer, S. Rezzonico, R. Hischier, R. Koehling, L. Thoeny-Meyer, and M. Zinn, *Isolation and Purification of Medium Chain Length Poly(3-hydroxyalkanoates) (mcl-PHA) for Medical Applications Using Nonchlorinated Solvents.* Biomacromolecules, 2010. **11**(10): p. 2716-2723.
119. Mantelatto, P.E., Durao N. A. S. , *Process for extracting and recovering polyhydroxyalkanoates (PHAs) from cellular biomass*, in *Patent 20080193987*, U.S., Editor 2008: USA.
120. Elbahloul, Y. and A. Steinbuechel, *Large-Scale Production of Poly(3-Hydroxyoctanoic Acid) by Pseudomonas putida GPo1 and a Simplified Downstream Process.* Applied and Environmental Microbiology, 2009. **75**(3): p. 643-651.
121. Ramsay, J.A., E. Berger, B.A. Ramsay, and C. Chavarie, *Recovery of poly-3-hydroxyalkanoic acid granules by a surfactant-hypochlorite treatment.* Biotechnology Techniques, 1990. **4**(4): p. 221-226.
122. Dong, Z. and X. Sun, *A new method of recovering polyhydroxyalkanoate fromAzotobacter chroococcum.* Chinese Science Bulletin, 2000. **45**(3): p. 252-256.
123. Holmes, P.A.L.G.B., *Separation process*, in *Patent 4910145*, U.S., Editor 1990: USA.

124. Yasotha, K., M.K. Aroua, K.B. Ramachandran, and I.K.P. Tan, *Recovery of medium-chain-length polyhydroxyalkanoates (PHAs) through enzymatic digestion treatments and ultrafiltration.* Biochemical Engineering Journal, 2006. **30**(3): p. 260-268.

125. Lakshman, K. and T.R. Shamala, *Extraction of polyhydroxyalkanoate from Sinorhizobium meliloti cells using Microbispora sp. culture and its enzymes.* Enzyme and Microbial Technology, 2006. **39**(7): p. 1471-1475.

126. Divyashree, M.S., T.R. Shamala, and N.K. Rastogi, *Isolation of polyhydroxyalkanoate from hydrolyzed cells of Bacillus flexus using aqueous two-phase system containing polyethylene glycol and phosphate.* Biotechnology and Bioprocess Engineering, 2009. **14**(4): p. 482-489.

127. Middelberg, A.P.J., *Process-scale disruption of microorganisms.* Biotechnology Advances, 1995. **13**(3): p. 491-551.

128. Kapritchkoff, F.M., A.P. Viotti, R.C.P. Alli, M. Zuccolo, J.G.C. Pradella, A.E. Maiorano, E.A. Miranda, and A. Bonomi, *Enzymatic recovery and purification of polyhydroxybutyrate produced by Ralstonia eutropha.* Journal of Biotechnology, 2006. **122**(4): p. 453-462.

129. Choi, J.-i. and S.Y. Lee, *Efficient and economical recovery of poly(3-hydroxybutyrate) from recombinant Escherichia coli by simple digestion with chemicals.* Biotechnology and Bioengineering, 1999. **62**(5): p. 546-553.

130. Lee, K.M., H.N. Chang, Y.K. Chang, B.S. Kim, and S.K. Hahn, *THE LYSIS OF GRAM-NEGATIVE ALCALIGENES-EUTROPHUS AND ALCALIGENES-LATUS BY PALMITOYL CARNITINE.* Biotechnology Techniques, 1993. **7**(4): p. 295-300.

131. Hahn, S.K., Y.K. Chang, and S.Y. Lee, *RECOVERY AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRIC ACID) SYNTHESIZED IN ALCALIGENES-EUTROPHUS AND RECOMBINANT ESCHERICHIA-COLI.* Applied and Environmental Microbiology, 1995. **61**(1): p. 34-39.

132. Berger, E., B.A. Ramsay, J.A. Ramsay, C. Chavarie, and G. Braunegg, *PHB recovery by hypochlorite digestion of non-PHB biomass.* Biotechnology Techniques, 1989. **3**(4): p. 227-232.

133. Chen, Y., H. Yang, Q. Zhou, J. Chen, and G. Gu, *Cleaner recovery of poly(3-hydroxybutyric acid) synthesized in Alcaligenes eutrophus.* Process Biochemistry, 2001. **36**(6): p. 501-506.

134. Kathiraser, Y., M.K. Aroua, K.B. Ramachandran, and I.K.P. Tan, *Chemical characterization of medium-chain-length polyhydroxyalkanoates (PHAs) recovered by enzymatic treatment and ultrafiltration.* Journal of Chemical Technology & Biotechnology, 2007. **82**(9): p. 847-855.

135. Harrison, S.T.L., *Bacterial cell disruption: A key unit operation in the recovery of intracellular products.* Biotechnology Advances, 1991. **9**(2): p. 217-240.

136. Bury, D., P. Jelen, and M. Kaláb, *Disruption of Lactobacillus delbrueckii ssp. bulgaricus 11842 cells for lactose hydrolysis in dairy products: a comparison of sonication, high-pressure homogenization and bead milling.* Innovative Food Science & Emerging Technologies, 2001. **2**(1): p. 23-29.

137. Park, P.K., E.Y. Kim, and K.H. Chu, *Chemical disruption of yeast cells for the isolation of carotenoid pigments.* Separation and Purification Technology, 2007. **53**(2): p. 148-152.

138. Balasundaram, B. and S.T.L. Harrison, *Influence of the extent of disruption of Bakers' yeast on protein adsorption in expanded beds.* Journal of Biotechnology, 2008. **133**(3): p. 360-369.

139. Geciova, J., D. Bury, and P. Jelen, *Methods for disruption of microbial cells for potential use in the dairy industry—a review.* International Dairy Journal, 2002. **12**(6): p. 541-553.

140. Kelly, W. and K. Muske, *Optimal operation of high-pressure homogenization for intracellular product recovery.* Bioprocess and Biosystems Engineering, 2004. **27**(1): p. 25-37.

141. Diels, A.M.J. and C.W. Michiels, *High-Pressure Homogenization as a Non-Thermal Technique for the Inactivation of Microorganisms.* Critical Reviews in Microbiology, 2006. **32**(4): p. 201-216.

142. Ghatnekar, M.S., J.S. Pai, and M. Ganesh, *Production and recovery of poly-3-hydroxybutyrate from Methylobacterium sp V49.* Journal of Chemical Technology & Biotechnology, 2002. **77**(4): p. 444-448.

143. Doucha, J. and K. Lívanský, *Influence of processing parameters on disintegration of Chlorella cells in various types of homogenizers.* Applied Microbiology and Biotechnology, 2008. **81**(3): p. 431-440.

144. Chen, G.Q., K.H. Konig, and R.M. Lafferty, *PRODUCTION OF POLY-D(-)-3-HYDROXYBUTYRATE AND POLY-D(-)-3-HYDROXYVALERATE BY STRAINS OF*

ALCALIGENES-LATUS. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 1991. **60**(1): p. 61-66.

145. Hänggi, U.J., *Pilot scale production of PHB with Alcaligenes latus*. Noves Biodegradable Microbial Polymers, ed. E.A. Dawes1990, Kluwer, Dordrecht.
146. Byrom, D., *PRODUCTION OF POLY-BETA-HYDROXYBUTYRATE - POLY-BETA-HYDROXYVALERATE COPOLYMERS.* Fems Microbiology Reviews, 1992. **9**(2-4): p. 247-250.
147. Chiellini, E. and R. Solaro, *Biodegradable Polymers and Plastics*2003, University of Pisa, Pisa, Italy: Springer. 395.
148. Chen, G.Q., G. Zhang, S.J. Park, and S.Y. Lee, *Industrial scale production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate).* Applied Microbiology and Biotechnology, 2001. **57**(1-2): p. 50-55.
149. Akiyama, M., T. Tsuge, and Y. Doi, *Environmental life cycle comparison of polyhydroxyalkanoates produced from renewable carbon resources by bacterial fermentation.* Polymer Degradation and Stability, 2003. **80**(1): p. 183-194.
150. Biby, G.D. *Degradable Polymers*. 04/10/2007 [cited 2013 06/01/2013]; Available from: http://www.icma.com/info/polymers.htm.
151. Clescerl, L.S.G., Arnold E. and Eaton, Andrew D., *Standard Methods for the examination of water and wastewater*. 20th ed ed1999, Washington DC: APHA.
152. Talon, O. and N. Berezina, *Method for rapid control of bacterial PHA production through thermogravimetric analysis.* Journal of Chemical Technology and Biotechnology, 2011. **86**(9): p. 1195-1197.
153. Serafim, L.S., P.C. Lemos, and M.A.M. Reis, *Effect of pH control on EBPR stability and efficiency.* Water Science and Technology, 2002. **46**(4-5): p. 179-184.
154. Merabet, S., D. Robert, J.-V. Weber, M. Bouhelassa, and S. Benkhanouche, *Photocatalytic degradation of indole in UV/TiO2: optimization and modelling using the response surface methodology (RSM).* Environmental Chemistry Letters, 2009. **7**(1): p. 45-49.
155. Costa, J.C. and M. Madalena Alves, *Posttreatment of Olive Mill Wastewater by Immobilized TiO2 Photocatalysis.* Photochemistry and Photobiology, 2013. **89**(3): p. 545-551.

156. *NIST/SEMATECH e-Handbook of Statistical Methods*. [cited 30/09/2013; Available from: http://www.itl.nist.gov/div898/handbook/.

157. Plasun, R., *Optimization of VLSI Semiconductor Devices*, in *Fakultät für Elektrotechnik*1999, Technischen Universität Wien Wien.

158. Vieira, M., *Estudo da cinética de acumulação e consumo de polímeros de reserva num reactor de biofilmes para a remoção do azoto*, in *Departamento de Engenharia Biológica*2005, Universidade do Minho: Braga.

159. Alvarez, A.F., H.M. Alvarez, R. Kalscheuer, M. Waeltermann, and A. Steinbuechel, *Cloning and characterization of a gene involved in triacylglycerol blosynthesis and identification of additional homologous genes in the oleaginous bacterium Rhodococcus opacus PD630.* Microbiology-Sgm, 2008. **154**: p. 2327-2335.

160. Revellame, E.D., R. Hernandez, W. French, W.E. Holmes, T.J. Benson, P.J. Pham, A. Forks, and R. Callahan Ii, *Lipid storage compounds in raw activated sludge microorganisms for biofuels and oleochemicals production.* RSC Advances, 2012. **2**(5): p. 2015-2031.

161. Sandra, P. and F. David, *A robust method for the determination of mineral oil in water samples(HYDROCARBON OIL INDEX).* Environmental Technology Online, 2004.

162. Papaneophytou, C. and D. Kyriakidis, *Optimization of Polyhydroxyalkanoates Production from Thermus thermophilus HB8 Using Response Surface Methodology.* Journal of Polymers and the Environment, 2012. **20**(3): p. 760-773.

163. Khanna, S. and A.K. Srivastava, *Statistical media optimization studies for growth and PHB production by Ralstonia eutropha.* Process Biochemistry, 2005. **40**(6): p. 2173-2182.

164. Hong, C., H. Hao, and W. Haiyun, *Process optimization for PHA production by activated sludge using response surface methodology.* Biomass and Bioenergy, 2009. **33**(4): p. 721-727.

165. Dalal, J., P.M. Sarma, A.K. Mandal, and B. Lal, *Response surface optimization of poly (3-hydroxyalkanoic acid) production using oleic acid as an alternative carbon source by Pseudomonas aeruginosa.* Biomass and Bioenergy, 2013. **54**(0): p. 67-76.

166. Basak, B., O. Ince, N. Artan, N. Yagci, and B. Ince, *Effect of nitrogen limitation on enrichment of activated sludge for PHA production.* Bioprocess and Biosystems Engineering, 2011. **34**(8): p. 1007-1016.

167. Mokhtarani, N., H. Ganjidoust, and E. Vasheghani Farahani, *Effect of process variables on the production of Polyhydroxyalkanoates by activated sludge.* Iranian Journal of Environmental Health Science & Engineering, 2012. **9**(1): p. 6.

168. Wang, Y.J., F.L. Hua, Y.F. Tsang, S.Y. Chan, S.N. Sin, H. Chua, P.H.F. Yu, and N.Q. Ren, *Synthesis of PHAs from waster under various C:N ratios.* Bioresource Technology, 2007. **98**(8): p. 1690-1693.

ANNEX

CALIBRATION CURVE OF HYDROCARBONS